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Studies on Amino Acids. IX.

On the Synthesis of α-Keto Acid Analogue of Methionine*

By Ichiro CHIBATA, Tasaburo KIGUCHI and Shun'ichi YAMADA

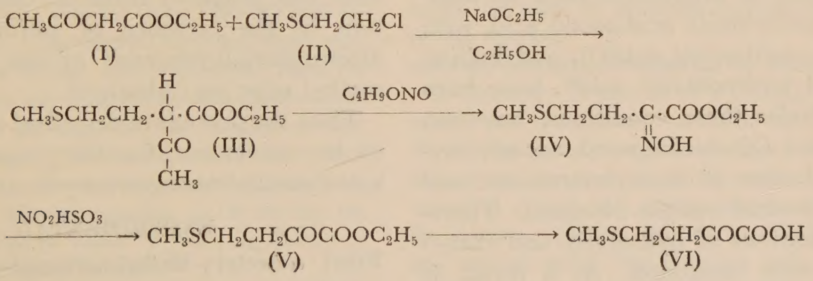
*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.***

Received May 24, 1957

The synthetic procedures for the preparation of α-keto acid analogue of methionine, α-keto-γ-methylmercaptobutyric acid, were investigated. As a result, the procedure consisting of the ester condensation between methyl β-methylmercaptopropionate and methyl oxalate, and subsequent hydrolysis and decarboxylation was found to be a satisfactory route.

In regard to amino acid metabolism, α-keto acids are considered very interesting substances. On the keto acid derived from methionine, α-keto-γ-methylmercaptobutyric acid (VI), several reports have been published. For instance, Cahill and Rudolph[1] observed that α-keto acid analogue of methionine resembles DL-methionine itself, effective in promoting the growth of rats fed with a diet low of this amino acid. Handler and Bernheim[2], reported that the α-keto derivative was as active as the natural amino acid in creatine synthesis by rat liver slices. Lampen et al.[3], also reported that the growth rate of a methionineless mutant of *E. coli* was approximately the same as that in the case of L-methionine and the inhibition of growth of the strain caused by norvaline was restored by the α-keto acid analogue of methionine.

On the preparation of the α-keto acid, biochemical procedures such as oxidation of methionine by liver slices[4] and by purified amino acid oxidase[5], have mainly been employed, but no satisfactory synthetic procedure has yet been published. For the purpose to obtain this substance in a larger amount, the synthetic chemical procedure is desirable. Therefore, in order to establish a convenient method of preparation the authors attempted the synthetic route, which had been employed for the preparation of the keto acids, as follows:



$$\underset{(I)}{CH_3COCH_2COOC_2H_5} + \underset{(II)}{CH_3SCH_2CH_2Cl} \xrightarrow[C_2H_5OH]{NaOC_2H_5}$$

$$CH_3SCH_2CH_2\cdot\overset{H}{\underset{\underset{CH_3}{CO}}{C}}\cdot COOC_2H_5\ (III) \xrightarrow{C_4H_9ONO} \underset{(IV)}{CH_3SCH_2CH_2\cdot\underset{NOH}{C}\cdot COOC_2H_5}$$

$$\xrightarrow{NO_2HSO_3} \underset{(V)}{CH_3SCH_2CH_2COCOOC_2H_5} \longrightarrow \underset{(VI)}{CH_3SCH_2CH_2COCOOH}$$

As a result, the keto acid was obtained, although, the yield of the reaction from isonitroso compound (IV) to the α-keto ester (V) was poor. Consequently, the route was not suitable for practical purposes.

---

* Presented at the Meeting of the Kansai Division of the Agricultural Chemical Society of Japan, Sakai, July 20, 1957.

** Honjo-kawasaki-cho, Oyodo-ku, Osaka.

1) W.M. Cahill and G.G. Rudolph, *J. Biol. Chem.*, **145**, 201 (1942).

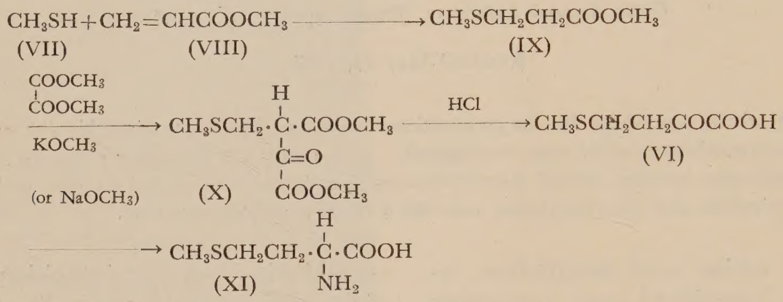
2) P. Handler and M.L.C. Bernheim, *J. Biol. Chem.*, **150**, 335 (1943).

3) J.O. Lampen, M.J. Jones and A.B. Perkins, *Arch. Biochem.*, **13**, 33 (1947).

4) H. Waelsch and E. Borek, *J. Am. Chem. Soc.*, **61**, 2252 (1939).

5) A. Meister, *J. Biol. Chem.*, **197**, 309 (1952).

Another route described in the following scheme was investigated, and a satisfactory result was obtained.



$$\underset{\text{(VII)}}{CH_3SH} + \underset{\text{(VIII)}}{CH_2{=}CHCOOCH_3} \longrightarrow \underset{\text{(IX)}}{CH_3SCH_2CH_2COOCH_3}$$

$$\xrightarrow[\substack{KOCH_3 \\ \text{(or } NaOCH_3\text{)}}]{\substack{COOCH_3 \\ | \\ COOCH_3}} \underset{\text{(X)}}{CH_3SCH_2\cdot\overset{\displaystyle H}{\underset{\substack{| \\ C=O \\ | \\ COOCH_3}}{C}}\cdot COOCH_3} \xrightarrow{HCl} \underset{\text{(VI)}}{CH_3SCH_2CH_2COCOOH}$$

$$\longrightarrow \underset{\text{(XI)}}{CH_3SCH_2CH_2\cdot\overset{\displaystyle H}{\underset{\substack{| \\ NH_2}}{C}}\cdot COOH}$$

Addition of methylmercaptan (VII) to methyl acrylate (VIII) gave methyl β-methylmercaptopropionate (IX), which was then condensed with methyl oxalate in the presence of potassium (or sodium) methoxide to give methyl α-keto-β-carbomethoxy-γ-methylmercaptobutyrate (X). This substance was a new compound and the potassium salt was obtained as a crystalline form, while the free ester was a viscous oily substance, which gave the 2,4-dinitrophenylhydrazone in yellow prisms, m.p. 115–117°.

As for the hydrolysis and decomposition of the derivatives of oxaloacetate to obtain keto acid, a number of procedures such as heating with hydrochloric acid-acetic acid, with hydrobromic acid-acetic acid[6,7], and boiling with diluted hydrochloric acid[8], have been reported. Under these conditions, however, the compound (X) decomposed, accompanying the evolution of methylmercaptan and the desired product was not obtained. Therefore the conditions of hydrolysis and decarboxylation were examined. As a result, it was found that heating of compound (X) with 5% hydrochloric acid at 70–75° accomplished the reaction. A mixture of reactants which at first formed two layers became homogeneous and the evolution of carbon dioxide was observed with proceeding of the reaction. The α-keto acid (VI) thus obtained, was an oily substance of which an aqueous solution was neutralized with sodium hydroxide, and acetone was added. The separated precipitate was recrystallized from water and methyl alcohol to yield the crystalline sodium salt, of which analysis agreed with calculated values. For further confirmation of the obtained α-keto acid (IV), 2,4-dinitrophenylhydrazone was prepared from the hydrochloric acid solution and paperchromatography and elemental analysis were performed. Furthermore, the 2,4-dinitrophenylhydrazone hydrogenated by tin in hydrochloric acid and the product was identified as being DL-methionine. When 2,4-dinitrophenylhydrazone was prepared from the methanol solution in the presence of sulfuric acid, 2,4-dinitrophenylhydrazone of the α-keto acid methyl ester was obtained.

Thus, the procedure described above seemed to be convenient for the preparation of α-keto-γ-methylmercaptobutyric acid.

EXPERIMENTAL

Ethyl α-Acetyl-γ-Methylmercaptobutyrate (III)[9,10,11]

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[6] P. Cordier, *Compt. rend.*, **228**, 848 (1949).

[7] J. Schreiber, *Compt. rend.*, **233**, 1039 (1951).

[8] F. Adickes and G. Andresen, *Ann.*, **555**, 41 (1944).

[9] J. Parrod and M. Rahier, *Compt. rend.*, **224**, 663 (1947).

[10] V.V. Feofilaktov and T.N. Ivanova, *Zhur. Obshchei Khim.* **21**, 1684 (1951); *C.A.* **46**, 3955 (1952).

[11] R. Riemschneider and A. Kluge, *Mh. Chem.*, **84**, 522 (1953); *C.Z.* **125**, 2154 (1954).

Metallic sodium (5.3 g) was dissolved in absolute alcohol (90 ml) and ethyl acetoacetate (I) (30 g) was added. To the above mixture, β-methylmercaptoethylchloride (II) (26 g) was added dropwise with stirring at 30–35°, and boiled on a water-bath for 3.5 hours. After standing overnight the separated sodium chloride was filtered off. The filtrate was concentrated and extracted with ether. The ethereal extract was dried over anhydrous sodium sulfate, evaporated, and the residue was distilled in vacuo to yield 26.4 g (56.1%) of III, b.p. 127–128°/5 mm.

Ethyl α-Isonitroso-γ-Methylmercaptobutyrate (IV)[9,12]

Metallic sodium (2.9 g) was dissolved in absolute alcohol (500 ml) and III (2.6 g) was added. The mixture was cooled to 10° and with stirring, *n*-butyl nitrite (14 g) was added dropwise for 20 minutes. Stirring was further continued for 2.5 hours at the same temperature, and the reaction mixture was then concentrated in vacuo. To the residue, a small amount of ice water was added and acidified with sulfuric acid. The separated oily substance was extracted with ether, and the ethereal layer was dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was distilled in vacuo to yield 18 g (75.0%) of IV, b.p. 143–145°/1.5 mm.

Ethyl α-Keto-γ-Methylmercaptobutyrate (V)[9]

To a solution of IV (18 g) dissolved in 83% formic acid (40 ml), nitrosylhydrosulfate (13 g) was added over the period of 50 minutes with stirring, while the temperature was kept below 3° during the addition. After the addition stirring was further continued until the temperature of the reaction mixture came to room temperature. At the end of the reaction, the dark red mixture turned to pale yellow. The reaction mixture was poured into ice water (100 ml) and extracted with ether. The ethereal layer was dried over anhydrous sodium sulfate, and evaporated. From the residue formic acid was removed by destillation under reduced pressure (20 mm), and the residue was then extracted with ether. The ethereal extract was washed with aqueous sodium bicarbonate, dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was distilled in vacuo to give 1.5 g (8.8%) of the product, b.p. 83–86°/2 mm.

2,4-Dinitrophenylhydrazone: Yellow needles, m.p. 116–117°. *Anal.* Calcd. for $C_{13}H_{18}O_6N_4S$: C, 43.57; H, 5.06; N, 15.64. Found: C, 43.84; H, 4.81; N, 16.03.

From other fractions, 3.3 g of IV was recovered.

Methyl β-Methylmercaptopropionate (IX)[13,14]

To a solution of benzene (100 ml) containing methylmercaptan (VII) (20 g) and sodium methoxide (0.4 g), methyl acrylate (VIII) (40 g) was added dropwise for 30 minutes with stirring at 5–8°. Stirring was further continued for 2 hours at the same temperatures. After standing overnight, the reaction mixture was filtered and concentrated. The residue was distilled in vacuo to yield 50 g (80.6%) of IX, b.p. 78–79°/14 mm.

Methyl α-Keto-β-Carbomethoxy-γ-Methylmercaptobutyrate (X)

To absolute ether (150 ml) potassium pellet (8 g) and absolute methanol (7 ml) were added and the mixture was boiled for 12 hours. To the resulting potassium methoxide, methyl oxalate (23 g) was added, and IX (26.8 g) was then gradually added with stirring. After addition the mixture was boiled for 6 hours. During the reaction, the mixture became gruel-like and yielded a white crystalline precipitate at the end of the reaction. The reaction mixture was allowed to stand overnight and the separated potassium salt of X was collected by filtration; yield 48 g (91.0%).

The salt was dissolved in cold water and acidified with hydrochloric acid. The separated oily substance was extracted with ether, the ether extract was washed with water, and dried over anhydrous sodium sulfate. Evaporation of the solvent left an orange-yellow oil of X, yield 23.5 g (53.4%).

2,4-Dinitrophenylhydrazone: Yellow prisms, m.p. 115–117°. *Anal.* Calcd. for $C_{14}H_{16}O_8N_4S$: C, 42.00; N, 4.03; N, 14.00. Found: C, 42.02; N, 4.24; N, 14.33. In place of potassium, sodium could be used in this reaction.

α-Keto-β-Methylmercaptobutyric Acid (VI)

i) From X– A mixture of X (23.5 g), conc. hydrochloric acid (47 ml) and water (230 ml) was stirred at 70–75° for 7 hours. The reaction mixture which formed two layers at the initial stage of the reaction turned to a clear pale yellow solution at the end of the reaction. Sodium chloride was added to the reaction mixture which was extracted with ether. The ethereal layer was washed with aqueous sodium bicar-

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[12] R.H. Barry and W.H. Hartung, *J. Org. Chem.*, **12**, 464 (1957).

[13] C.D. Hurd and L.L. Gershbein, *J. Am. Chem. Soc.*, **69**, 2328 (1947).

[14] J.L. Szabo and E.T. Stiller, *J. Am. Chem. Soc.*, **70** 3667 (1948).

bonate to dissolve the acidic substance. The aqueous layer was washed with ether, acidified with hydrochloric acid, salted out with sodium chloride, and extracted with ether. The ether extract was washed with water and dried over anhydrous sodium sulfate. Evaporation of ether left viscous oil of VI; yield 9.6 g (60.8%).

Sodium salt: VI was dissolved in water and adjusted to pH 6.8 with sodium hydroxide. After the solution was allowed to stand overnight, acetone was then added, and the separated precipitate was collected by filtration. Recrystallization from water, and subsequent recrystallization from methanol yielded colorless pillars. *Anal.* Calcd. for $C_5H_7O_3SNa \cdot \frac{1}{2}H_2O$: C, 33.39; H, 4.53. Found: C, 33.51; H, 4.50.

2,4-Dinitrophenylhydrazone: The substance from the dilute hydrochloric acid solution was recrystallized from methanol-acetic acid mixture to give yellow needles; m.p. 150–151°. *Anal.* Calcd. for $C_{12}H_{14}N_4O_6S$: C, 40.25; H, 3.69; N, 17.07. Found: C, 40.15; H, 3.94; N, 16.82.

Paperchromatography of the 2,4-dinitrophenylhydrazone was carried out by the ascending method employing *n*-butanol saturated with 3% aqueous ammonia as a developing solvent. The obtained R_F value was 0.62 (13°, Toyo filter paper No. 50) which agrees with the value reported[15].

When 2,4-dinitrophenylhydrazone was prepared from the methanol-sulfuric acid solution, esterification occurred and 2,4-dinitrophenylhydrazone of methyl α-keto-γ-methylmercaptobutyrate was obtained as yellow needles, m.p. 168–169°. *Anal.* Calcd. for $C_{11}H_{14}N_4O_6S$: C, 42.11; H, 4.12, N, 16.37. Found: C, 41.95; H, 4.11; N, 16.04.

ii) From V– To the alcoholic sodium hydroxide solution (3 ml) prepared from alcohol (3 ml), sodium (0.14 g) and water (0.11 ml), V (1 g) was added. The reaction mixture became turbid and separated precipitate. After standing overnight in a refrigerator, the separated sodium salt was collected; yield 0.8 g (88.8%). Recrystallization as described in the case of i) gave colorless pillars.

DL-Methionine (XI)

For further confirmation of the above mentioned product, the 2,4-dinitrophenylhydrazone of IV was dissolved in acetic acid and hydrogenated by tin powder and hydrochloric acid. Paperchromatography of the hydrogenated product was carried out for identification. The ascending method at 13° with Toyo filter paper No. 50, was performed and the spots were located by spraying ninhydrin reagent. When pyridine-water was used as a developing solvent, R_F values for the hydrogenated product and an authentic sample of DL-methionine were 0.35 and 0.36, respectively. Both samples gave the same R_F value of 0.45, when *n*-butanol-acetic acid-water (4 : 1 : 1) (V/V/V) was employed. In both developing solvents, the mixture of an authentic sample of DL-methionine and the hydrogenated product gave a single spot. As a result of the paperchromatography, the hydrogenated product of the 2,4-dinitrophenylhydrazone was identified as to be DL-methionine.

Acknowledgements. The authors are grateful to Prof. S. Sugasawa of University of Tokyo, Prof. S. Akabori of Osaka University, Prof. H. Mitsuda of Kyoto University and Dr. M. Fujisawa, Diretor of this Laboratory, for their helpful advice and encouragement rendered during the course of this work.

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[15] D. Cavallini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1949).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 337~339, 1957]

Studies on the Metabolic Products of *Macrosporium porri* Elliott

Part II. Structure of Macrosporin (Group I)

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Institute of Dairy Chemistry, Rakuno College, Nopporo, Hokkaido

Received June 17, 1957

Macrosporin $C_{16}H_{12}O_5$, a metabolic product of *Macrosporium porri* Elliott, has been proved to be mono-methyl-mono-methoxy-di-hydroxy-anthraquinone, owing to the fact that macrosporin monomethylether was oxidized with CrO_3 to 5-methyl 4-methoxy phthalic acid.

Previously work[1] of this series reports on a new orange-yellow pigment $C_{16}H_{12}O_5$ isolated from the laboratory cultures of *Macrosporium porri* Elliott, the pathogenic organism of stone-leek black spot disease. This pigment which was named "macrosporin", had two hydroxyl groups and one methoxy group. The present paper deals with the structure of macrosporin.

From the color reaction of magnesium-acetate, its solubility in alkali, and ultra-violet absorption spectra, this pigment was considered to be a polyhydroxyanthraquinone. In further experiments macrosporin was methylated employing CH_2N_2 to macrosporin monomethylether $C_{16}H_{11}O_5OCH_3$, m.p. 209–210°, which seems to show two hydroxyl groups in macrosporin at α and β positions respectively. This methylation is owing to a well known and generally accepted belief that the hydroxyl group at α position of polyhydroxyanthraquinone is hardly methylated with CH_2N_2. Macrosporin monomethylether was oxidized with CrO_3 in the mixture of glacial acetic acid and acetic anhydride to 5-methyl-4-methoxy-phthalic acid, which was found to be identical with an authentic specimen by both the mixed melting point method and infra-red absorption spectra. This means that macrosporin is a monomethyl-monomethoxy dihydroxy-anthraquinone.

Also the infra-red absorption spectrum of macrosporin gave a non-chelated C=O band at 6.02 μ, a chelated band at 6.11 μ, and a broad OH band at 3.04 μ[1]. The 6.11 μ band is assigned to the C=O group chelated with the α-hydroxyl group[2]. The chelated C=O band at 6.11 μ and OH band at 3.04 μ did not appear in both diacetate and dimethylether which retained the non-chelated C=O band at 6.04 μ and 6.08 μ, respectively. Both the chelated C=O band at 6.14 μ and the non-chelated C=O band at 5.99 μ were, however, found in the spectrum of macrosporin monomethylether. This suggests that α-position hydroxyl group is retained. These results also lead to the conclusion that macrosporin has both α and β position hydroxyl groups. Ninety per cent sulphuric acid at 100°, usually demethylates methoxy groups at the α-position of methoxyanthraquinone, but not at the β-position. As macrosporin was found to be not demethylated in the above conditions, the methoxy group in it is considered to be at the β-position.

Phycion (7-methoxy-emodin), a metabolic product of *Asp. glaucus*, is the only pigment to be found in nature, having a constitution of monomethyl-monomethoxy-dihydroxy anthraquinone. Macrosporin is believed to be

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[1] R. Suemitsu et al., This Bulletin, **21**, 1 (1957).

[2] M. St., C. Flett, *J. Chem. Soc.*, **1948**, 1441.

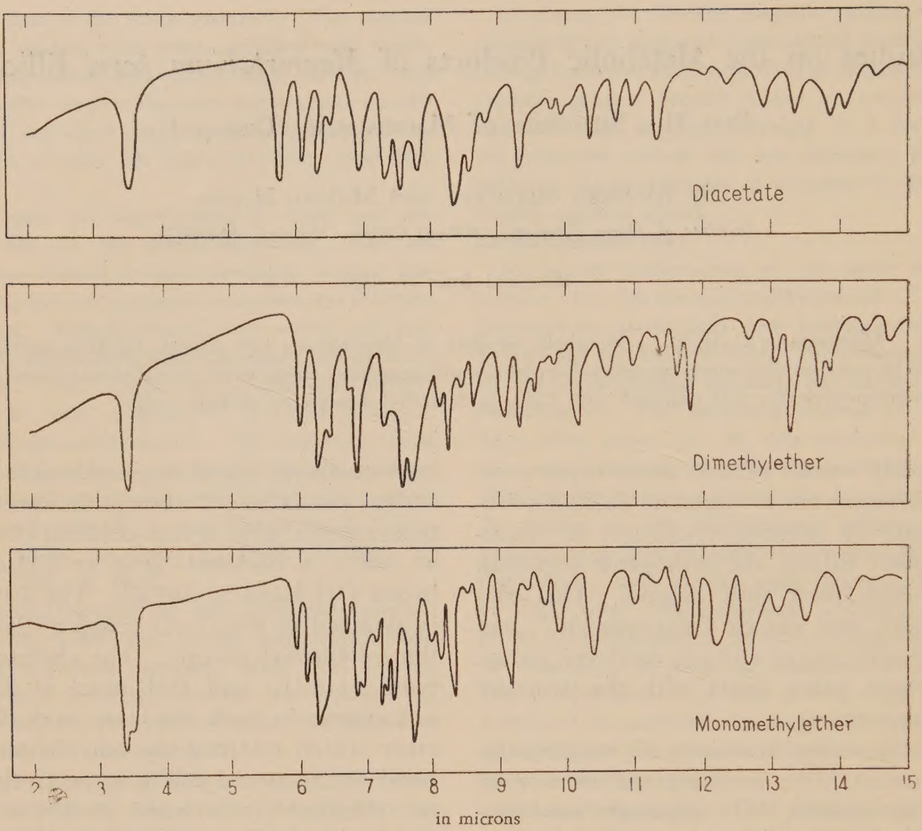

FIG. 1. Infra-red Absorption Spectra of Diacetate, Dimethylether and Monomethylether Derivatives from Macrosporin (Nujol mull).

an isomer of phycion. The result of further studies will be published in the near further.

EXPERIMENTAL

Macrosporin Monomethylether Macrosporin (650 mg) was dissolved in the mixture of ether and acetone and an excess of an ethereal solution of CH_2N_2 was added. The orange colored solution immediately became brown-yellow, and there was a feeble evolution of nitrogen. After it was allowed to stand overnight, the solvent was removed to a yellow matter, which was recrystallized from glacial acetic acid to give fine yellow needles (300 mg), m.p. 208–209°. *Anal.* Found: C, 68.25; H, 5.00; OCH_3, 20.73; Calcd. for $C_{16}H_{11}O_4OCH_3$: C,68. 45; H, 4.73; OCH_3 20.81.

Oxidation of Macrosporin Monomethylether Macrosporin monomethylether (320 mg) was dissolved in a mixture of glacial acetic acid (20 ml) and acetic anhydride (40 ml) at 100° and, while maintaining this temperature, a solution of CrO_3 (1 g) in water (1 ml) and glacial acetic acid (10 ml) was added with stirring for 20 min.. Heating and stirring were continued for further 10 min.. The dark green reaction mixture was cooled, evaporated in vacuo to a syrup, to which dil. HCl (50 ml) was added. The solution, now containing crystals, was extracted with ether and the ethereal solution was shaken with 5% Na_2CO_3 solution, after acidification, which was extracted with ether, and removal of the solvent, yielded crude crystals (120 mg). This material was recrystallized from water to white needles, m.p. 176° (decom.). *Anal.* Found: C, 56.77; H, 5.11; OCH_3, 15.16. Calcd. for $C_9H_7O_4$-OCH_3: C, 57.14; H, 5.80; OCH_3, 14.76.

This specimen was not depressed by the admixture of synthetic 5-methyl-4-methoxy-phthalic acid, m.p.

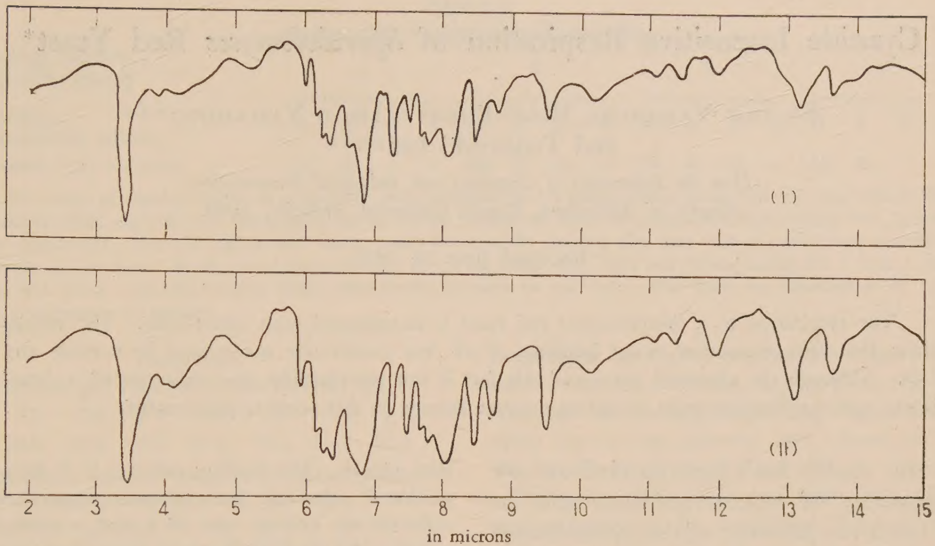

FIG. 2. Infra-red Absorption Spectra of a Oxidation Product of Monomethylether (I) and Synthetic 5-Methyl-4-methoxy-phthalic Acid (II) (Nujol mull).

176–177°. The infra-red absorption spectra were also found to be quite similar.

Charlesworth[3] reported 5-methyl-4-methoxy-phthalic acid as colorless crystals, m.p. 166.5–167° (decom.). Raistric et al.,[4] reported of an acid which they claimed to be 5-methyl-4-methoxy phthalic acid m.p. 180–180.5° as a degradation product of dihydroglaciolic acid, and they oxidized it to 5-methoxybenzene-1,2,4-tricarboxylic acid, which was proved to be identical with the synthetic specimen. We adopted the method of Charlesworth and obtained the same acid m.p. 166°, which he had described. As this substance, however, seemed to be a mixture of 5-methyl-4-methoxy-phthalic acid and 5-methoxybenzene 1,2,4-tricarboxylic acid, the following experiments were carried on.

The substance (420 mg) was refluxed at 140° with acetic anhydride for 1 hour. The solvent was removed in vacuo and the residue was recrystallized from *n*-hexane to give white needles (100 mg), m.p. 157–158° (Raistrick gave m.p. 158.5–159° of 5-methyl-4-methoxy-phthalic anhydride). This substance (40 mg) was dissolved in water (2 ml), boiled for 5 min. and cooled. The fine white needles separated were filtered and recrystallized from water, m.p. 176–177°. *Anal.* Found: C, 57.18; H, 5.08; Calcd. for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80.

This synthetic 5-methyl-4-methoxy-phthalic acid of m.p. 176–177° is believed to be an authentic one, although its melting point was 3–4 degrees lower than that of Raistrick.

Acknowledgement. We are indebted to Prof. S. Takei, Prof. T. Mitsui and Dr. M. Nakajima, of Kyoto Univ. for their valuable suggestions. Grateful thanks are also due to Prof. Y. Obata, Prof. H. Igarashi of Hokkaido Univ. for their advices and encouragement and Mr. Z. Kumazawa of Kyoto Univ. for the infra-red spectra measurements.

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3) Charlesworth, et al., *Can. J. Research*, **23B**, 17 (1945).

4) Raistrick et al., *Biochem. J.*, **50**, 610 (1952).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 340~345, 1957]

Cyanide Insensitive Respiration of *Sporobolomyces* Red Yeast*

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and Tadayoshi IDE****

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Received June 18, 1957

The respiration of a *Sporobolomyces* red yeast is investigated with intact cells. The results show that the respiration is not inhibited at all, but moderately accelerated by cyanide and CO. Although the chemical nature of this fact is not yet clarified, the existence of a heat-labile and cyanide-insensitive oxidative enzyme system in this yeast is presumable.

Numerous studies have been carried out on the respiration of microorganisms and in most of them the presence of the cytochrome-cytochrome oxidase system has been demonstrated. However in some cases, complete insensitivity of the respiration to cyanide, even in the presence of substrate, has been observed. Gerard1) has shown that the oxygen consumption of *Sarcina lutea* is not inhibited by 10^{-2} mole of sodium cyanide and this is also true of the increased respiration evoked by glucose. Yamaguchi2) has obtained similar results with *Micrococcus citreus* and *Mic. ochraceus*, and emphasized the significance of the pigments of these organisms.

EXPERIMENTS AND RESULTS

Sporobolomyces ruberrimus which has been described in 1950³⁾ was used in this work. Red yeast cells used for manometric studies were grown, being shaken with excess air, in a liquid medium containing 5 per cent sucrose, 0.5 per cent peptone, 0.1 per cent KH_2PO_4, 0.1 per cent $NaNO_3$ and 0.05 per cent $MgSO_4 \cdot 7H_2O$ (tap water). The shaking culture, in 2 days at 25°, produced relatively homogeneous suspension of red cells in the average size of $4\mu \times 8\mu$, multiplying up to about 70 times of the inoculated seed. The cells were harvested by centrifugation, washed three times with 0.9 per cent saline. They were resuspended in 0.2 M phosphate buffer (pH 5.8) and mechanically shaken for another 24-hour period at 25° to diminish the endogenous respiration as much as possible. The starved cells thus obtained were centrifugated and washed again. Then, after being suspended in the same phosphate buffer, the cells were ready for experiments.

Oxygen uptake was measured by the usual Warburg manometric technique at 25° and the results were expressed in Q_{O_2} values. All results appearing in this paper represent values obtained from at least duplicate determinations.

Lack of Anaerobiosis As previously reported3), this red yeast is strictly aerobic and is not provided with an emergency mechanism to induce fermentation in anaerobic conditions. The Pasteur reaction fails in this yeast. So, as shown in Table I, the yeast can not survive in anaerobic conditions and dies away in either 2 or 3 days. On the contrary, if shaken in aerobic conditions, in spite of no nutrient supplement, the yeast can continue to live for at least ten days.

Effect of pH The optimum pH for glucose oxidation of the red yeast was found to be approximately 5.8. In the alkaline side the oxidation rate decreased markedly (Table II).

Effect of NaCN NaCN, in concentrations up to 0.02 M did not inhibit the oxygen uptake of this yeast in the least degree, but accelerated it moderately. This

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1) R.W. Gerard, *Biol. Bull.*, **60**, 227 (1931).

2) S. Yamaguchi, *Acta Phytochimica*, **8**, 157 (1934).

3) I. Yamasaki and H. Fujii, *J. Agr. Chem. Soc. Japan*, **24**, 11 (1950).

TABLE I

EFFECT OF ANAEROBIC CONDITIONS

Shaking culture, aerobic (days)	1				2				3				4			
Shaking, anaerobic (days)	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Growth (+) on slant	⧻	±	−	−	⧻	+	±	−	⧻	+	±	−	⧻	+	−	−

In the course of shaking culture in several lots, each composed of 3-4 culture flasks, usually 250-ml long necked flasks containing 50 ml medium with inoculum, a lot of culture is taken off from the culture, one after another in 1, 2, 3 or 4 days, respectively, from the shaker, and cotton plugs are promptly changed with gum ones in a steril-state in order to close the culture flasks. In the closed state, they are set on the shaker once more and shaken further for 1, 2 or 3 days. From each 1,2 or 3 days-long shaken flasks, some streaks are made on agar slants, these slants are incubated at 25° for 3 days to determine the growth.

TABLE II

OPTIMUM pH FOR GLUCOSE OXIDATION

pH	1.3	2.0	4.4	5.8	7.0	7.8	9.6
Q_{O_2}	18.6	23.3	24.2	25.6	20.2	14.4	10.4

Each manometer vessel of about ten ml-content contains: 0.5 ml of cell suspension (about 5-6 mg, dry weight) in 0.2 M phosphate buffer at various pH, 0.2ml of 0.5 M glucose and distilled water to bring the volume to 1.0ml and 0.3 ml of 10 per cent KOH is placed in the center well. Total volume is 1.3 ml, 30-minute measurements. Endogenous respiration Q_{O_2} = 8.59 (pH 5.8)

TABLE III

EFFECT OF CYANIDE ON RESPIRATION OF YEAST OF VARIOUS CULTIVATION AGES

	Age of culture (days)	2	4	6	14
Q_{O_2}	Endogenous	8.16	7.00	5.85	4.02
	Glucose (A)	36.8	23.7	20.0	17.0
	Glucose+NaCN (B)	46.7	31.5	29.9	34.4
	Ratio (B/A), per cent	127	133	150	202

Each vessel has 0.5 ml of cell suspension (5-8 mg, dry weight) in 0.2 M phosphate buffer at pH 5 8, 0.3 ml of 0.3 M glucose and 0.2 ml water or 0.2 ml of 0.01 M NaCN. Center well contains 0.3 ml of 10 per cent KOH. Total volume 1.3 ml, 30-minute measurements.

remarkable fact was observed not only with NaCN, but also with KCN and HCN.

As shown in Table III, Q_{O_2} value, both endogenous and with glucose, gradually decreased inversely proportional to the length of culture. On the other hand, the ratio, Q_{O_2} value with cyanide to that of without cyanide, increased just proportional to the length of cultivation. These were true of the endogenous respiration, too. Therefore, a 2-day culture was mostly used in the experiments.

Effect of *p*-Phenylendiamine (Paramine) The Nadi reagent and paramine are generally employed for the indirect testing of cytochrome oxidase. The Nadi reaction, applied to this yeast suspension, after Keilin's original method[4], was distinctly positive. Concerning paramine two ways of observation were made: one, the oxidation of paramine; the other, its effect on the oxygen uptake in respiration; and in either case in the presence and absence of cyanide. The results are given in Table IV.

From the table, it is remarkable that, in the presence of paramine, the respiration of intact cells, both endogenous and with glucose, increases considerably and remains increased even with cyanide. On the contrary, with warmed cells, which thereby became fully sensitive to cyanide, the accelerating effect of paramine on respiration drops off completely in the presence of cyanide.

But the oxidation of paramine—which is recognized by violet colorization—was completely inhibited by cyanide in all cases. A stronger colorization was observed by warming the cells, as shown by Keilin.

From these results, the presence and the functioning of cytochrome oxidase in this yeast is to be convincible. If this is the case, another oxydative enzyme system, responsible for cyanide-insensitive oxygen uptake, should be functioning in this yeast respiration.

Effect of Heat Treatment *Sporobolomyces* yeasts, hitherto described, are generally known to be mesophilic and their temperature maximum for growth lies between 27° and 33°. Corresponding to this fact,

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[4] D. Keilin, *Proc. Roy. Soc.*, **B**, **104**, 206 (1929).

TABLE IV
EFFECT OF PARAMINE ON O_2 UPTAKE

Substrate	Treatment	Control Q_{O_2}	NaCN Q_{O_2}	Paramine		Paramine+NaCN	
				Q_{O_2}	Colour change in 30 min.	Q_{O_2}	Colour change in 30 min.
None (Endogenous)	Intact cell	8.12	18.0	23.4	Light violet	24.5	Colourless
	Warmed cell (45°, 15 min.)	3.18	0.79	8.15	Dark violet	1.16	Colourless
Glucose	Intact cell	30.5	35.0	36.4	Light violet	34.1	Colourless
	Warmed cell (45°, 15 min.)	14.3	4.62	18.4	Dark violet	5.15	Colourless

Conditions are similar to those in Table III.
Final concentration: 0.1 M glucose, 0.002 M NaCN, 0.01 M paramine (neutralized with HCl), 0.1 M phosphate buffer (pH 5.8)

the respiratory system of this red yeast was found to be sensitive towards temperature. The cells suspended in phosphate buffer (pH 5.8) were warmed for 15 minutes at various temperatures and their effects on respiration were observed by measuring the Q_{O_2}. The results are given in Table V.

TABLE V
EFFECT OF HEAT TREATMENT ON YEAST RESPIRATION WITH OR WITHOUT CYANIDE

Heat treatment (15 min.)	Q_{O_2}			
	Warmed cell		Intact cell (control)	
	Glucose	Glucose +NaCN	Glucose	Glucose +NaCN
38°	22.8	20.8	33.4	46.7
43°	18.5	11.6	28.4	38.1
45°	21.1	5.20	36.8	46.5

Conditions are similar to those of Table III.
Final concentration: 0.1 M glucose, 0.002 M NaCN, 0.1 M phosphate buffer (pH 5.8)

After heat treatment at 45° for 15 minutes, the subsequent respiration decreased to about a half of the initial and became fully sensitive to cyanide. So, residual respiration with cyanide was only from one-tenth to one-thirtieth of the normal intact cell (Fig. 1).

From these results (Table IV and Fig. 1), it is evident that the postulated cyanide-insensitive enzyme system is much more heat-labile than the cytochrome oxidase.

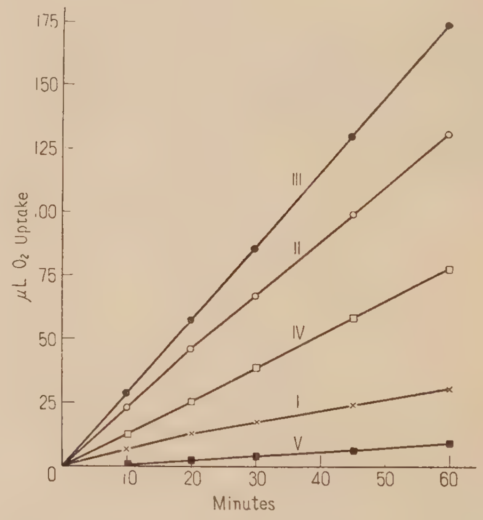

FIG. 1. Effect of Cyanide on the Intact and the Warmed Cell (45°, 15 min.).

Conditions same as in Table V.
Intact cell: I. No addition. II. 0.1 M Glucose. III. 0.1 M Glucose+0.002 M NaCN
Warmed cell: IV. 0.1 M Glucose. V. 0.1 M Glucose+0.002 M NaCN.

Effect of Oxygen Pressure Gas mixtures consisting of 20, 17, 13, 10, 7 and 4 per cent O_2 and 80, 83, 87, 90, 93 and 96 per cent N_2 respectively, were prepared by mixing suitable quantities of pure gases. In these gas mixtures, the effect of varying partial pressures of oxygen on the respiration of this yeast was studied. Fig. 2 shows oxygen pressure-O_2 uptake curves with or without cyanide. It can be said that the oxygen uptake with cyanide is markedly more sensitive to lower partial pressure of oxygen than the normal respiration without cyanide. Ten per cent O_2 atmosphere caused an inhibition of 16 per cent with cyanide, whilst it caused 6 per cent inhibition without it; and in 4 per cent O_2 atmosphere, the inhibi-

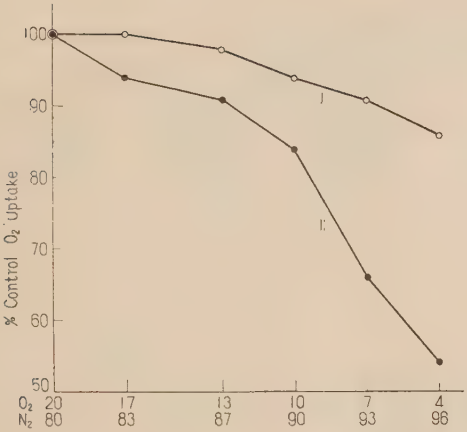

FIG. 2. Effect of Lower Oxygen Pressures on Respiration (Expressed in % of the Normal Value).
Conditions same as in Table III. Yeast cels 5-8 mg.
I. without cyanide Q_{O_2} in air 34.2.
II. with 0.002M NaCN Q_{O_2} in air 43.5.

tion rate rose up to 46 per cent with cyanide, but only 14 per cent without it.

Effect of Carbon Monoxide It is well known that cytochrome oxidase is inhibited by carbon monoxide as it is by cyanide, but this inhibition can be released by strong illuminations. To observe the response of this yeast respiration to carbon monoxide in the dark and in the light, Warburg vessels containing a gas-mixture of 90 per cent CO and 10 per cent O_2 were exposed, in a covered water bath at 25°, to two 100-watt tungsten lamps, immersed in the bath, at a distance of 5 to 10 cm from the manometer vessels. The lamps were turned on at intervals of 15 minutes. Typical results are given in Table VI.

It was shown that the oxygen uptake in the glucose oxidation increases 26–27 per cent in the CO atmosphere in the dark, and this increment disappears in the light. These values of increment coincide well with those given by the addition of cyanide.

Effect of Cultural Conditions It is of interest to observe that the insensitivity of this respiration to cyanide alters owing to the aerobiosis of the cells. It

TABLE VI

EFFECT OF LIGHT ON CO INHIBITION

Experiment No.	90% N_2+10% O_2 (Control)		90% CO+10% O_2			
			Dark		Light	
	Q_{O_2}	Ratio	Q_{O_2}	Ratio	Q_{O_2}	Ratio
1	29.3	100	36.9	126	27.1	97
2	26.6	100	34.5	127	29.0	112

Conditions are same as those of Table III.
Yeast cells, 5-8 mg. Glucose concentration, 0.1 M

TABLE VII

EFFECT OF CULTURAL CONDITIONS

Exp. No.	Cluture method	Days of culture	Resting in phosphate buffer	Q_{O_2}		
				Endogenous	Glucose	Glucose+NaCN
1.	Shaking culture	2	Shaking, 24 hrs.	8.92	33.4	46.7
2.	Shaking culture	2	Settled, 24 hrs.	12.0	22.4	18.0
3.	Surface culture in liquid medium	15	None	15.5	26.0	23.0
4.	Slant culture on agar medium	2	None	12.8	20.4	17.1

No. 1—Shaking highly aerobic culture as described in the manuscript.
No. 2—Cells, grown in the above conditions, are kept in a settled state, instead of shaking, in phosphate buffer for a further 24 hr-period.
No. 3—Surface culture in a 500-ml Erlenmeyer flask containing 100 ml of the same medium.
No. 4—Same medium as above solidified by 2% agar.

TABLE VIII

BALANCE OF GLUCOSE BURNED AND OXYGEN CONSUMED

	Measured				Calculated			
	R.Q.	Glucose disappearing (A) μM	O_2-consumed μl	O_2-consumed μM	Completely oxidized glucose (B) μM	O_2-consumed / Glucose disappearing	Glucose disappearing / Glucose burned (A/B)	Glucose assimilated / Glucose burned
Without NaCN	0.97	10	220	10	10/6	1	6	5
With NaCN	0.94	10	670	30	30/6	3	2	1

Conditions are as described in Fig. 3. Glucose 10 μM.

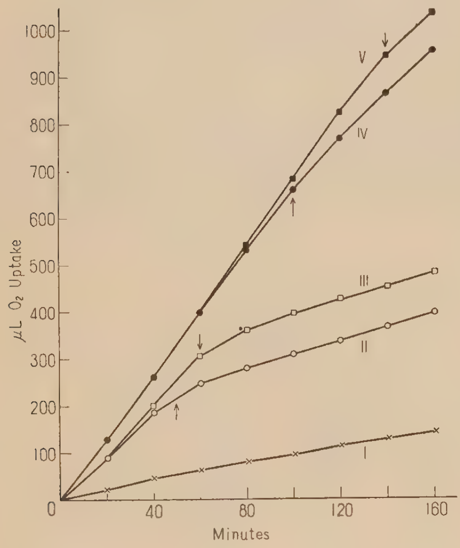

FIG. 3. Balance of Glucose and Oxygen.

Each vessel contains: 1 cc. of cell suspension in 0.2 M phosphate buffer (pH 5.8), 0.2 cc. of distilled water or 0.2 cc. of 0.02 M NaCN and 0.8 cc. of glucose in various concentration. Center well contains 0.3 cc. of 10% KOH. Total volume 2.3 cc.

I. Endogenous respiration.
II. 10 μM Glucose.
III. 13.4 μM Glucose.
IV. 10 μM Glucose, 0.002 M NaCN.
V. 13.4 μM Glucose, 0.002 M NaCN

develops most markedly in a highly aerobic shaking culture such as shown above and not so vividly in usual surface or streak culture. And, such insensitivity once gained, becomes weak when the cells are kept in a settled state in medium or in a buffer a forbout twenty-four hrs. (Table VII).

Balance of Glucose Used and Oxygen Uptake Applying so minute and definite concentration of glucose that complete burning is accomplished within 150 minutes, the quantitative relation between sugar used and oxygen uptake in the respiration of this yeast were determined. The results are shown in Fig. 3 and Table VIII.

The respiratory quotient was found to be 0.97 (≑1) for the glucose respiration without cyanide, and 0.94 (≑1) with cyanide. Therefore glucose was burned completely in both cases.

But the ratio of moles of oxygen consumed to moles of glucose disappearing in the medium, was found to be ca. 1 when cyanide was not used, whilst it was ca. 3 when cyanide was added. Since the complete oxidation of 1 mole glucose requires 6 moles oxygen of theory and the carbohydrate breakdown by this red yeast runs exclusively along the pathway of aerobic oxidation, it can be estimated that this yeast assimilates about five moles of glucose for each mole of glucose burned in the normal respiration, but in respiration with cyanide, only one mole glucose for each mole glucose burned.

This denotes the fact that respiration with cyanide is of a quite different biochemical nature from the normal respiration of this organism: in short, the efficiency of respiration in the former case is much inferior to that in the latter case.

On the other hand, cultural experiments showed that in the presence of cyanide the cells cannot propagate in the least, but die away in almost twenty-four hrs.. So the lethal effect of cyanide on the yeast must not be due to specific inhibition of the cytochrome-cytochrome oxidase system, but to some other general poisonous action.

DISCUSSION

This red yeast contains carotenoid pigments in a large amount[5], so it is incapable of observing the bands of the cytochrome spectrum directly in the yeast cell*. But the positive reactions with Nadi and paramine reagents, and the complete inhibition of oxygen uptake by the cyanide in the warmed cells will be enough to convince us of the existence and the functioning of the cytochrome oxidase in this yeast respiration. Another evidence for the presence of cytochrome oxidase in yeast is the fact that the effect of carbon monoxide is completely reversed by light.

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[5] I. Yamasaki, S. Morisita and M. Kikei, This Bulletin, 17, 86 (1941).

The increased respiration under the influence of cyanide or carbon monoxide suggests 3 assumptions: (1) it may merely be a result of the stimulation by these reagents; (2) the inhibitory effect of cyanide on the catalase may cause an apparent increase of oxygen consumption; (3) some enzyme system other than cytochrome oxidase may be functioning in the respiration.

The first assumption is evidently wrong because of the results indicated in Fig. 1, Fig. 2, Table VI and Table VII.

The second assumption is eliminated by the fact that the cyanide in the concentration of 0.002 M cannot completely arrest the catalase activity of these intact yeast cells.

The third assumption is strongly supported by the results shown above. And this postulated oxydative enzyme system is insensitive to cyanide and CO, but it is sensitive to heat (Fig. 1) and to lower oxygen pressure (Fig. 2) as in the case of the old yellow enzyme[6]. Indeed, the presence of such a cyanide- or/and carbon monoxide-insensitive enzyme system has lately been reported in yeast[7], in higher plants[8,9] and in animals[10].

But, since the red yeast can not propagate under conditions with cyanide, the physiological significance of respiration insensitive to cyanide and CO is open to further studies.

SUMMARY

1) The respiration of a *Sporobolomyces* red yeast which is strictly aerobic, is not inhibited at all, but accelerated moderately by the addition of cyanide or CO.

2) The presence and functioning of cytochrome oxidase is demonstrated by the oxidation of the Nadi reagent and paramine, and photo-reversibility of the effect of CO.

3) Besides cytochrome oxidase, the existence of another oxidative enzyme system which is cyanide- and carbon monoxide-insensitive, but sensitive to heat and to lower oxygen pressure, is presumed.

4) This enzyme system develops in the yeast most markedly in highly aerobic shaking culture and diminishes in anaerobic conditions.

5) The red yeast neither grows nor survives in the medium in the presence of cyanide, despite the accelerated respiration caused thereby.

6) The molar ratio, oxygen consumed to glucose burned, in respiration is ca. 1 without cyanide, whilst ca. 3 with cyanide.

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* The colourless mutant of *Sp. ruberrimus* which was designated as *Sp. ruberrimus var. albus*, has been obtained[3], and by this strain, the presence and the functioning of cytochrome bands, just as well as in the case of baker's yeast, were recognized with the help of a microspectroscope.

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[7] T. Sawai, *Botanical Magazine, Tokyo*, **66**, 34 (1952).
[8] H. Levy and A.L. Shade, *Arch. Bioch.*, **19**, 273 (1948).
[9] G.G. Laties, *Arch. Bioch.*, **27**, 404 (1950).
[10] W.A. Robbie, *J. General Physiology*, **32**, 655 (1949).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 346~350, 1957]

Microbiological Studies of *Coli-aerogenes* Bacteria*

Part III. Oxidations of Glucose and Pyruvic Acid

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The oxidative degradation of glucose by *coli-aerogenes* bacteria was investigated: a remarkable yield (more than 0.5 mole per one mole of glucose) of α-ketoglutaric acid was obtained with suspensions of washed cells of the bacteria grown aerobically on glucose medium, and pyruvic acid fermentation was revealed in the presence of arsenic compounds not only with growing culture but also with the washed cells. Moreover, pyruvic acid was found to be a potent precursor of α-ketoglutaric acid.

INTRODUCTION

In the previous paper[1], the authors have made investigations on the fermentation of glucose by the bacteria of *coli-aerogenes* types and it was found that when the bacteria were cultivated under aerobic conditions in replacement of usual anaerobic incubation, a large amount of α-ketoglutaric acid was obtained in place of lactic acid as the major product of fermentation. From the results of the authors' experiments, with various kinds of *coli-aerogenes* bacteria, there were also pointed out so many types of their oxidative fermentations, such as α-ketoglutaric acid-, pyruvic acid-, acetic, pyruvic (acetoine) and α-ketoglutaric acids-, acetic and α-ketoglutaric acids-fermentations and the complete oxidation of glucose into CO_2 and H_2O.

Koepsell et al., investigated the production of pyruvic and α-ketoglutaric acids in oxidative fermentations of gluconic and 2-ketogluconic acids by *Pseudomonas fluorescens* and suggested that α-ketoglutaric acid might arise from the condensation of pyruvic acid with fragments such as C_2-or C_3 compounds produced during fermentation, since the accumulation of pyruvic acid was decreased against the increasing yield of α-ketoglutaric acid at the later stage of growing culture of the bacterium.

In the present paper, various investigations were instituted with glucose and pyruvic acid in order to ascertain the yield of α-ketoglutaric acid, and to ascertain whether pyruvic acid would be obtained in the oxidation of glucose by the bacteria of *coli-aerogenes* types, and also to ascertain whether such a three-carbon compound as pyruvic acid would be potent in the production of α-ketoglutaric acid.

EXPERIMENTAL AND RESULTS

Production of α-Ketoglutaric Acid from Glucose by Washed Cells of *E. Coli*

In the present paper experiments on the production of α-ketoglutaric acid from glucose were carried out with washed cells of *E. coli* grown aerobically on glucose medium. One hundred ml of a medium containing 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.3% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.2% NaCl was taken in a 500 ml shaking flask and sterilized $CaCO_3$ of the amount corresponding to 50% of glucose was added in a flask at the time of inoculation. The bouillon culture of *E. coli*, obtained by 1–2 days' incubation at 37°C under static conditions was used as

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* A part of this paper was read before the annual meeting of the Japanese Biochemical Society (November, 1955).

[1] H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 210, 215 (1957).

TABLE I
YIELD OF α-KETOGLUTARIC ACID FROM GLUCOSE BY WASHED CELLS OF *E. COLI* GROWN ON GLUCOSE MEDIUM

Bacterial cells (mg)	Time of incubation (hours)	Addition of CaCO$_3$ (mg)	Initial conc. of glucose (μM)	Glucose consumed (μM)	α-Ketoglutaric acid produced	
					(μM)	(Molar yield)
41	13	0	1000	—	228	0.23
41	13	0	1000	—	253	0.25
48	16	0	1000	—	203	0.20
63	15	0	1000	—	216	0.22
38	16	0	1000	—	310	0.31
46	11	100	1000	—	577	0.58
51	13	100	1000	—	612	0.61
43	13	100	1000	753	410	0.54
43	13	100	1000	787	312	0.40
36	15	100	1000	842	600	0.71
51	14	100	1000	1000	762	0.76

an inoculum and the flask was placed on a shaker. After incubation at 30°C for 2 days, the bacterial cells were collected by a centrifuge and washed with distilled water. The bacterial cells were then suspended in distilled water and the concentration was determined by the optical density, observed with an electrophotometer. The reaction mixtures (5–20 ml) containing 1 mM glucose, 1 mM phosphate buffer (pH: 6.2), 10 μM MgSO$_4$, 0–100 mg CaCO$_3$ and the suspensions of washed cells, were dispensed in a 100 ml test tube or a 500 ml shaking flask, and kept aerobically on the shaker at 28–30°C. After incubation, the bacterial cells were removed by a centrifuge and then the amounts of consumed glucose and producd α-ketonic acids (α-ketoglutaric and pyruvic) were determined by the methods mentioned in the previous paper[1].

From the results shown in Table I, it can be recognized that α-ketoglutaric acid was produced without any accumulation of pyruvic acid in the course of fermentation of glucose by the washed cells of *E. coli* grown on glucose medium and that the yields of α-ketoglutaric acid exceeded 0.5 mole (40.6 g acid from 100 g glucose) from one mole of glucose, which would often be observed with growing culture reported in the previous paper.

The fact that α-ketoglutaric acid is produced more than 0.5 mole from one mole of glucose has also been ascertained in the growing cultures of microorganisms other than *coli-aerogenes* by Koepsell et al.[3], Masuo et al.[4], Asai et al.[5] and Doi et al.[6]. The predicted theoretical yield of α-ketoglutaric acid would be 0.5 mole per mole of consumed glucose according to the Warburg-Lipmann-Dickens pathway in which oxidation of glucose-6-phosphate to 5-phosphopentose via 6-phosphogluconate, thence, 6-phospho-2-ketogluconate has been suggested. This one-carbon degradation of the glucose molecule is considered to be repeated until one mole of triose phosphate and three moles of carbon dioxide are generated.

Although the existence of the above pathway in the bacteria belonging to *E. coli* has already been pointed out by some workers[7], the results of our experiments from which such a high yield of α-ketoglutaric acid as more than 0.5 mole per one mole of consumed glucose is ascertained, suggest that such alternative pathways relating to the Embden-Meyerhof scheme or $C_6 \rightarrow C_5 \rightarrow C_2 + C_3$ would exist besides the Warburg-Lipmann-Dickens scheme.

Conversion of α-Ketoglutaric Acid Fermentation to Pyruvic Acid Fermentation in Presence of Arsenite or Arsenate

Experiments of Growing Culture. Arsenite is a

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[2] H.J. Koepsell, *J. Biol. Chem.*, **186**, 743 (1950).

[3] H.J. Koepsell, F.H. Stodola and E.S. Sharpe, *J. Am. Chem. Soc.*, **74**, 5142 (1952).

[4] E. Masuo and Y. Wakizaka, *J. Agr. Chem. Soc. Japan*, **29**, 546 (1955); *ibid.*, **29**, 550 (1955).

[5] T. Asai, K. Aida. Z. Sugisaki and N. Yakeishi, *J. Gen. Appl. Microbiol.*, **1**, 308 (1955).

[6] S. Doi et al., Reported at the meeting of the Agricultural Chemical Society of Japan held in 1956 in Ise City.

[7] S.S. Cohen et al., *J. Biol. Chem.*, **188**, 509 (1951); *Fed. Proc.*, **10**, 173 (1951); *Biochem J.*, **55**, 23 (1953); *ibid.*, **55**, 33 (1953)

well known inhibitor of the oxidative decarboxylation of α-ketoglutaric acid to succinic acid and carbon dioxide[5)8)9)]. In the present paper, oxidative fermentations of *E. coli* were at first, instituted on a shaker with a medium containing 10% glucose, 0.1% $(NH_4)_2HPO_4$, 0.1% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 5% $CaCO_3$ with or without Na-arsenite which was added after 1 day's fermentation, in order to ascertain what influence would be exerted by arsenite on the production of α-ketoglutaric acid from glucose with the growing culture of the bacterium.

TABLE II

INFLUENCE OF Na-ARSENITE ON OXIDATION OF GLUCOSE IN GROWING CULTURE OF *E. COLI*

Addition of Na-arsenite (M/l)	0	2×10^{-3}	4×10^{-3}
Glucose consumed (mM/dl)	44.0	42.6	50.5
α-Ketoglutaric acid produced (mM/dl)	20.5	16.4	15.0
(Molar yield)	0.47	0.39	0.30
Pyruvic acid produced (mM/dl)	0	12.5	25.5
(Molar yield)		0.29	0.51
Acetic acid produced (mM/dl)	0	2.34	4.84
(Molar yield)		0.05	0.09

It will be seen in Table II that such a remarkable production of pyruvic acid took place, that the maximum yield amounted about 0.51 mole per one mole glucose consumed, while the accumulation of α-ketoglutaric acid (Yield being about 0.30 mole) was considerably decreased when the bacterium was cultivated under aerobic conditions in the presence of 4×10^{-3} M Na-arsenite.

Similar experiments were carried out with the media containing 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4 and other mineral matters in either the presence or absence of Na-arsenate. The results of the experiments are given in Table III, from which pyruvic acid fermentation was again observed to have taken place, instead of α-ketoglutaric acid fermentation, with glucose medium containing Na-arsenate, although the yields of pyruvic as well as α-ketoglutaric acids varied with the concentration of arsenic compound.

TABLE III

INFLUENCE OF Na-ARSENATE ON OXIDATION OF GLUCOSE IN GROWING CULTURE OF *E. COLI*

Addition of Na-arsenate (M/l)	0	8.1×10^{-3}	2.4×10^{-2}
Glucose consumed (mM/dl)	7.87	7.60	7.87
α-Ketoglutaric acid produced (mM/dl)	4.78	3.43	1.71
(Molar yield)	0.61	0.45	0.22
Pyruvic acid produced (mM/dl)	0	2.84	6.84
(Molar yield)		0.37	0.87

Experiments with Suspensions of Washed Cells of *E. Coli*. The influences of arsenate upon oxidative fermentation of glucose were further investigated in the experiments with the washed cells of *E. coli* grown aerobically at 30°C for 2 days on 2% glucose medium in which the same mineral matters were employed as in the experiments shown in Table I. Table IV shows the results of the experiments, which were instituted as follows: the reaction mixture (7.5 ml) contained 0.5–1.0 mM phosphate buffer (pH: 6.2), 10 μM $MgSO_4$ 150 mg $CaCO_3$, 1 mM glucose, and the requisite amounts of washed cells and of Na-arsenate which were illustrated in the Table, fermenta-

TABLE IV

INFLUENCE OF Na-ARSENATE UPON OXIDATION OF GLUCOSE BY SUSPENSION OF WASHED CELLS OF *E. COLI*

Exp. No.	Bacterial cells (mg)	Addition of Na-arsenate (μM)	Glucose consumed (μM)	α-Ketoglutaric acid produced		Pyruvic acid produced	
				(μM)	(Molar yield)	(μM)	(Molar yield)
Ia	43	0	842	600	0.71	0	
Ib	43	484	1000	270	0.27	702	0.70
Ic	43	648	1000	243	0.24	800	0.80
Id	43	970	1000	204	0.20	926	0.93
IIa	51	0	1000	762	0.76	0	
IIb	51	162	1000	572	0.57	270	0.27
IIc	51	162	1000	448	0.45	557	0.56
IId	51	970	835	312	0.37	550	0.66

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8) T.K. Walker et al., *Nature.* **168**, 1042 (1951).

9) D.J.D. Hockenhull et al., *ibid.*, **168**, 1043 (1951).

tions being carried out on the shaker at 30°C for 14 hours. It will be seen from Table IV that pyruvic acid was again produced remarkably from glucose by the washed cells in presence of higher concentration of Na-arsenate with which the production of α-ketoglutaric acid was extremely decreased. Therefore, the influence of arsenate upon the kinds of oxidation products obtained with washed bacterial cells was found to be the same as those observed with growing culture of the bacterium. The results of observations hitherto mentioned suggest the possibility that the action of arsenic compounds such as arsenite or arsenate would inhibit pyruvic oxidase to cause a conversion of α-ketoglutaric acid fermentation to pyruvic acid fermentation and pyruvic acid would be a potent intermediate substance in the production of α-ketoglutaric acid, since, the amount of increasing pyruvic acid were found to be nearly equivalent to those of decreasing α-ketoglutaric acid revealed by the inhibitors.

Manometric Experiments. The conventional Warburg technique was used for the determination of oxygen and carbon dioxide in the following manner: the reaction mixture (2.5 ml) was composed of 300 µM phosphate buffer (pH: 6.0), 100 µM glucose, 2 µM $MgSO_4$ and the washed cells (29 mg on dry basis) of *E. coli* grown on glucose medium and incubation was carried out in either the presence or absence of Na-arsenate (320 µM) at 30°C in air. As can be seen in Fig. 1, the evolution of carbon dioxide was remarkably decreased in presence of Na-arsenate but considerable difference of oxygen uptake was not pointed out by the inhibitor, so that the R.Q. values calculated were found to be about 0.5–0.65 and about 1.0 in the presence and absence of arsenate respectively. These results, therefore, show that some portion of the oxygen consumed during aerobic decomposition of glucose would be attributed to some other reactions not involving oxidative decarboxylation. In the case of pyruvic acid fermentation, it is considered that some portion of molecular oxygen could be consumed through the reaction chain composed of diaphorase, cytochrome and cytochrome oxidase, following the coupled reaction between the reduction of coenzyme and oxidation of phosphoglyceraldehyde to phosphoglyceric acid.

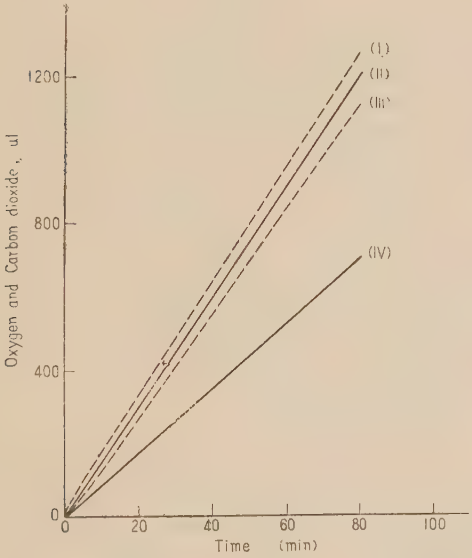

FIG. 1. Effect of Na-arsenate upon Oxidation of Glucose by Washed Cells of *E. coli*.

I. Glucose (Oxygen uptake).
II. Glucose (Carbon dioxide evolution).
III. Glucose + Na-arsenate (Oxygen uptake).
IV. Glucose + Na-arsenate (Carbon dioxide evolution).

Production of α-Ketoglutaric Acid from Pyruvic Acid by Washed Cells of *E. Coli*

The authors' interest has been directed to pyruvic acid which might be supposed to be an intermediate substance in the formation of α-ketoglutaric acid from glucose according to the experimental results obtained by Koepsell et al., and also by the authors. The following experiments were at first instituted in order to verify the synthesis of α-ketoglutaric acid from pyruvic acid by washed cells of *E. coli* with which the production of α-ketoglutaric acid from glucose was

TABLE V

PRODUCTION OF α-KETOGLUTARIC ACID FROM PYRUVIC ACID BY WASHED CELLS OF *E. COLI* GROWN ON GLUCOSE MEDIUM

Bacterial cells (mg)	Time of incubation (hours)	Pyruvic acid consumed (µM)	α-Ketoglutaric acid produced (µM)	(Molar yield)
240	13	4000	773	0.194
240	13	2000	204	0.102
245	15	4000	1170	0.293
245	15	2000	400	0.200
200	15	4000	383	0.096
335	16	4000	216	0.054
148	13	4000	1335	0.334
145	8	2000	593	0.296
175	14	4000	450	0.113

TABLE VI

INFLUENCE OF CONCENTRATIONS OF WASHED CELLS OF *COLI-AEROGENES* UPON YIELD OF α-KETOGLUTARIC ACID OBTAINED FROM PYRUVIC ACID

Exp. No.	Bacterial cells (mg)	Time of incubation (hours)	Pyruvic acid consumed (μM)	α-Ketoglutaric acid produced	
				(μM)	(Molar yield)
Ia	145	14	4000	1160	0.290
Ib	435	14	4000	493	0.123
IIa	148	14	4000	1335	0.334
IIb	444	14	4000	830	0.207

ascertained as was already mentioned. The bacterial cells were collected by a centrifuge and then suspended in distilled water after being washed. The reaction mixture (40 ml) contained 5 mM phosphate buffer (pH: 6.2), 100 μM $MgSO_4$, requisite amounts of Na-pyruvate and bacterial cells as are illustrated in Table V, and incubated aerobically on the shaker at 30°C. The results of the experiments are given in Table V, from which washed cells of *E. coli* grown aerobically on glucose medium have been found to be capable of producing α-ketoglutaric acid from Na-pyruvate. The production of α-ketoglutaric acid from pyruvic acid would necessarily involve carbon synthesis ($C_3 \rightarrow C_5$) and yet α-ketoglutaric acid was obtained with such high yields as shown in Table V. It is a well known fact that pyruvic acid is metabolized aerobically by the bacteria of coli-aerogenes types to acetic acid[10], CO_2, H_2O and to cell materials[11], namely, various reactions including acetic acid fermentation, complete oxidation (normal respiration) and oxidative assimilation would take place by the bacteria. However, from the experimental results obtained by the authors it is recognized that α-ketoglutaric acid is the major oxidation product of pyruvic acid, although the yields of α-ketoglutaric acid from pyruvate were observed to be extremely dependent upon the experimental conditions as will be seen in Table VI in which better yields of α-ketoglutaric acid were obtained at a higher ratio of pyruvic acid/bacterial cells. These experimental results correspond to those of growing cultures observed in the previous paper in which good yields of α-ketoglutaric acid from glucose were obtained at a higher ratio of carbon/nitrogen, while at a lower ratio, the yield of α-ketoglutaric acid was exceedingly decreased.

SUMMARY

1. With washed cells of the bacteria of *coli-aerogenes* grown aerobically on glucose medium, the yield of α-ketoglutaric acid from glucose was found to exceed 0.5 mole per one mole of glucose consumed, and the mode of oxidative degradation of glucose was discussed.

2. In presence of arsenic compounds, it was observed that the oxidative fermentation of glucose with either washed cells or growing culture of *E. coli* gave rise to pyruvic acid as a major product, while the evolution of carbon dioxide was remarkably decreased and the mode of oxidation of glucose was again discussed from the standpoint of pyruvic acid fermentation.

3. It was found that α-ketoglutaric acid was obtained in a high yield (0.5–0.7 mole from two moles of pyruvic acid) when the washed cells were incubated on the shaker with a Na-pyruvate solution. According to the experimental conditions, a remarkable difference was pointed out among the yields of α-ketoglutaric acid.

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[10] J.L. Still, *Biochem. J*, **35**, 380 (1941).

[11] C.E. Clifton, *Advances in Enzym.*, **6**, 269 (1946).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 351~356, 1957]

Microbiological Studies of *Coli-aerogenes* Bacteria*

Part IV. Production of α-Ketoglutaric Acid

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The production of α-ketoglutaric acid by the bacteria of *coli-aerogenes* was observed to be inhibited in the presence of αα′-dipyridyl or *o*-phenanthroline. From experimental results it was concluded that iron might be a sort of cofactor of the enzyme system producing α-ketoglutaric acid. Moreover, α-ketoglutaric acid was found to be produced not only from lactic acid by either growing culture or the washed cells, but also from pyruvic acid under enzymatic conditions such as dried cells or cell-free extracts.

INTRODUCTION

In the previous paper[1)], the authors have suggested that pyruvic acid would be a potent precursor of α-ketoglutaric acid, since the accumulation of α-ketoglutaric acid by the bacteria of *coli-aerogenes* was remarkably decreased against the increasing yield of pyruvic acid in presence of inhibitors such as arsenic compounds. Moreover, it was verified by the authors that the oxidative fermentations of pyruvic acid with washed cells of the bacteria grown aerobically on glucose medium give rise to a large quantity of α-ketoglutaric acid under suitable conditions. These results indicate that α-ketoglutaric acid is not directly produced by the one carbon degradation of glucose [glucose $(C_6)\ \xrightarrow{-CO_2}$ α-ketoglutaric acid (C_5)], but it would be synthesized from pyruvic acid which is produced from glucose by the fermentation.

In the present paper, experiments were at first carried out in order to ascertain whether there was any effect of iron on α-ketoglutaric acid fermentation. Subsequently, oxidative fermentation of three carbon compounds such as pyruvic and lactic acids were investigated.

EXPERIMENTAL AND RESULTS

Inhibiting Action of αα′-Dipyridyl or *o*-Phenanthroline upon Production of α-Ketoglutaric Acid by *Coli-aerogenes*

Koepsell investigated oxidations of gluconic and 2-ketogluconic acids by *Pseudomonas fluorescens* and found that the oxidation of 2-ketogluconic acid was remarkably accelerated in the presence of a trace (1 p.p.m.) of inorganic iron such as ferrous ammonium sulfate[2)]. From this, it is anticipated that the production of α-ketoglutaric acid from glucose with *E. coli* will be inhibited by αα′-dipyridyl, which has the ability of forming its Fe-complex compound, if inorganic iron might be indispensable to the α-ketoglutaric acid producing system. Therefore, experiments were carried out with *E. coli* in the following manner. After being grown with a shaker at 30°C for 2 days in a medium containing 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.3% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl and 1% $CaCO_3$, *E. coli* was collected by a centrifuge and washed with distilled water. The reaction mixture (7.0 ml) containing 1 mM phosphate buffer (pH: 6.2) 10 μM $MgSO_4$, 1 mM glucose and 38 mg bacterial cells was taken up in a 100 ml test tube or a 500 ml shaking flask and kept aerobically on a shaker at 28°C for 16 hours in the presence or absence of $\alpha\alpha'$-dipyridyl, $\alpha\alpha'$-dipyridyl and $FeSO_4$. After incubation, bacterial cells were removed by a centrifuge and then amounts of α-ketonic acids produced were determined by the methods mentioned in the previous paper[3]. As will be seen in Table I, $\alpha\alpha'$-dipyridyl had a remarkable inhibiting effect upon the production of α-ketoglutaric acid from glucose, but its inhibiting action was reduced to some extent by the addition of $FeSO_4$.

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* A part of this paper was read before the annual meeting of the Agricultural Chemical Society of Japan (March, 1956).

1) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 346 (1957).

2) H.J. Koepsell, *J. Biol. Chem.*, **186**, 743 (1950).

In order to ascertain whether $\alpha\alpha'$-dipyridyl or *o*-phenanthroline would reveal any noticeable effect on the production of α-ketoglutaric acid from pyruvate by the bacteria of *coli-aerogenes* types, experiments were at first instituted with washed cells of *E. coli*, grown aerobically on a 2% glucose medium in which the concentrations of the mineral matters were chosen in the same amounts as those mentioned in the experiments given in Table I. Incubation was carried out on the shaker at 30°C for 14 hours in the presence or absence of inhibitors on the reaction mixture (40 ml) containing 5 mM phosphate buffer (pH: 6.0), 100 μM $MgSO_4$ 3.5–4.0 mM Na-pyruvate and 140 mg bacterial cells. From the results given in Table II (A), it was found that the consumption of pyruvic acid was not greatly affected at the end of incubation, but the production of α-ketoglutaric acid was extremely inhibited in the presence of $\alpha\alpha'$-dipyridyl or *o*-phenanthroline. The same experiments as performed with *E. coli* were instituted with *B. lactis aerogenes* (No. B–2) as follows: the reaction mixture (25 ml) containing 1 mM phosphate buffer (pH: 6.0), 60 μM $MgSO_4$ 4.0 mM Na-pyruvate and 80 mg washed cells of the bacterium grown aerobically on a 2% glucose medium, was incubated on the shaker at 30°C for 14 hours in the presence or absence of *o*-phenthroline. Table II (B) shows that the amount of α-ketoglutaric acid produced by *B. lactis aerogenes* was again remarkably decreased in the presence of *o*-phenanthroline, however, the formations of acetic acid and acetoine could proceed. Thus, it is found that inorganic iron would be indispensable to a certain factor of the synthesizing system of α-ketoglutaric acid from pyruvate, while the production of acetic acid or acetoine from pyruvate would proceed even on the conditions deficient in inorganic iron. These conclusions were further ascertained from the following experiments with washed cells of *E. coli* grown aerobically on 2% glucose medium in the same manner as

TABLE I

INFLUENCE OF $\alpha\alpha'$-DIPYRIDYL ON PRODUCTION OF α-KETOGLUTARIC ACID FROM GLUCOSE BY WASHED CELLS OF *E. COLI* GROWN AEROBICALLY ON GLUCOSE MEDIUM

Exp. No.	1	2	3
$\alpha\alpha'$-Dipyridyl added, (μM/7 ml)	0	10	5
$FeSO_4$ added, (μM/7 ml)	0	0	20
Glucose used (mM)	1.000	1.000	1.000
α-ketoglutaric acid produced (mM)	0.310	Trace	0.200

TABLE II

INFLUENCE OF $\alpha\alpha'$-DIPYRIDYL AND *o*-PHENANTHROLINE ON PRODUCTION OF α-KETOGLUTARIC ACID FROM PYRUVIC ACID BY WASHED CELLS OF *COLI-AEROGENES* TYPES GROWN AEROBICALLY ON GLUCOSE MEDIUM

(A) *E. Coli*

Addition of inhibitors,	$\alpha\alpha'$-Dipyridyl		*o*-Phenanthroline	
(μM/40 ml)	0	30	0	25
Pyruvic acid initial (mM)	3.500	3.500	4.000	4.000
Pyruvic acid consumed (mM)	3.477	3.432	4.000	4.000
α-Ketoglutaric acid produced (mM)	0.927	0	0.815	0.142
Acetic acid produced (mM)	0	2.950	0	3.210

(B) *B. lactis aerogenes*

o-Phenanthroline added (μM/25 ml)	0	25
Pyruvic acid initial (mM)	4.000	4.000
Pyruvic acid consumed (mM)	4.000	4.000
α-Ketoglutaric acid produced (mM)	0.660	Trace
Acetic acid produced (mM)	0.900	1.880
Acetoine produced (mM)	Trace	0.340

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[3] H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 210 (1957).

already mentioned. The washed cell suspensions (15 ml) containing 2.5 mM phosphate buffer (pH: 6.2) and 130 mg bacterial cells, were preincubated in the presence or absence of 25 μM *o*-phenanthroline, under static conditions, at 37°C for 1 hour and thereafter, 4 mM Na-pyruvate, 30 μM $MgSO_4$ and 0–100 μM $FeSO_4$ were added to the mixture and the volume made up with water to 27 ml and then the solution was analysed after being incubated on the shaker at 30°C for 13 hours.

TABLE III

ACTION OF INORGANIC IRON UPON BACTERIAL CELLS POISONED BY *o*-PHENANTHROLINE

Exp. NO.	1	2	3	4
o-Phenanthroline added (μM/27 ml)	0	25	25	25
$FeSO_4$ added (μM/27 ml)	0	0	50	100
Pyruvic acid consumed (mM)	4.000	4.000	4.000	4.000
α-Ketoglutaric acid produced (mM)	1.132	0.203	0.518	0.568
Acetic acid produced (mM)	0	3.120	1.600	1.500

TABLE IV

PRODUCTION OF α-KETOGLUTARIC ACID FROM PYRUVIC ACID BY WASHED CELLS OF *COLI-AEROGENES* BACTERIA GROWN AEROBICALLY ON Fe RICH MEDIUM

Bacterial cells, (mg)	Time of incubation, (hours)	Pyruvic acid consumed, (mM)	α-Ketoglutaric acid produced, (Molar yield)
145	12	4.000	0.300
138	14	4.000	0.204
138	14	4.000	0.278
92	7	2.000	0.272

It will be seen in Table III that the inhibiting action of *o*-phenanthroline could be reduced to some extent by the addition of inorganic iron. Since inorganic iron was ascertained to be indispensable for the production of α-ketoglutaric acid as was mentioned above, the following experiments on the oxidative fermentation of pyruvic acid were carried out with washed cells of the bacteria, grown aerobically for 2 days at 30°C in an iron-rich medium which contained 5 mg % $FeSO_4 \cdot 7H_2O$.

It will be seen in Table IV that the results of the experiments were highly reproducible, and that pyruvic acid was observed to give rise to a large quantity of α-ketoglutaric acid, when the fermentations were carried out at 30°C on the shaker with the reaction mixture (40 ml) containing 5 mM phosphate buffer (pH: 6.0), 100 μM $MgSO_4$, 4–2 mM Na-pyruvate and about 140–100 mg Fe-rich bacterial cells.

Production of α-Ketoglutaric Acid from Pyruvic Acid by Dried Cells of *E. Coli*

The following experiments were carried out with Na-pyruvate, in order to investigate whether a remarkable yield of α-ketoglutaric acid obtained with washed intact cells of *E. Coli* would again be attainable with its dried cells.

E. coli, which was grown aerobically on the same 2% glucose medium as already mentioned, was harvested by a centrifuge, washed with water and then dried by an electric fan at room temperature for 5–7 hours. After being suspended in water, the dried cells were added to the solution containing 5 mM phosphate buffer (pH: 6.2), 4–2 mM Na-pyruvate and 100 μM $MgSO_4$, and after the volume of reaction mixture was made with water to 40 ml, fermentations were carried out on the shaker at 30°C. Results of the experiments are given in Table V, from which it will be seen that the dried cells of *E. coli* grown on glucose medium are capable of synthesizing α-ketoglutaric acid from pyruvate and the yield of α-ketoglutaric acid was ascertained to be 0.35–0.40 mole from 2 moles of pyruvic acid consumed after 6–14 hours' incubation.

Production of α-Ketoglutaric Acid from Pyruvic Acid by Cell-Free Extracts of *E. Coli*

TABLE V

PRODUCTION OF α-KETOGLUTARIC ACID FROM PYRUVIC ACID BY DRIED CELLS OF *E. COLI* GROWN AEROBICALLY ON GLUCOSE MEDIUM

Dried cells, (mg)	Time of incubation, (hours)	Pyruvic acid consumed, (mM)	α-Ketoglutaric acid produced	
			(mM)	(Molar yield)
200	6	1.640	+	
200	14	4.000	0.700	0.175
195	14	4.000	0.815	0.204

With the washed or dried cells of *E. coli*, pyruvic acid is the most potent precursor in the production of α-ketoglutaric acid as already mentioned. Consequently, the following investigations were made in order to ascertain the synthesis of α-ketoglutaric acid from pyruvate by the cell-free extracts of *E. coli* grown aerobically on a 2% glucose medium in the same manner as already mentioned. After being harvested by a centrifuge, the bacterial cells were frozen and ground in a mortar with dry-ice powder. Phosphate buffer (pH: 6.0) was added to the raptured bacterial cells obtained by freezing and thawing procedures[4] shown in Table VI and after being kept at 0 C for several hours, the solution was centrifuged at 4500 r.p.m. to remove the cellular debris. The reaction mixture composed of 5 mM phosphate buffer (pH: 6.0), 40–50 μM $MgSO_4$, 1.6 mM Na-pyruvate and cell-free extracts, the volume was made up with water to 40–50 ml and incubation was carried out on the shaker at 30°C for 13 hours.

TABLE VI

PREPARATION OF CELL-FREE EXTRACTS

Washed cells
↓ added dry ice powder
  frozen by mixing and grinding
Frozen cell powder
↓ thawed on water bath
Thawed cell paste
↓ added dry ice powder
Frozen cell powder
↓ thawed, extracted with phosphate buffer, centrifuged
Cell-free extracts

From Table VII it will be seen that α-ketoglutaric acid was again produced from pyruvate with the cell-free extracts, although its activity was observed to be lower than that of the washed or dried cells.

Production of α-Ketoglutaric Acid from Lactic Acid

The bacteria of *coli-aerogenes* types are well known to produce lactic acid as their major end-product under anaerobic conditions in which pyruvic acid is revealed as a hydrogen acceptor in the reoxidation of reduced coenzyme.

On the other hand, it is obvious from the experimental results obtained by the authors that pyruvic acid is most potent in producing α-ketoglutaric acid with the bacteria of *coli-aerogenes* types. From these results it is of interest to ascertain what kind of oxidative metabolism of the bacteria would take place when lactic acid is employed instead of pyruvic acid. Experiments were at first carried out with washed cells of the bacteria. The reaction mixtures which were composed of 1–10 mM phosphate buffer (pH: 6.0), 60–300 μM $MgSO_4$ 2–12 mM Na-L-lactate and washed cells of *E. coli* grown aerobically on 2% glucose medium containing 5 mg % $FeSO_4$ in the same manner as was already mentioned, were incubated on the shaker at 30°C for 7 hours. Table VIII shows that under aerobic conditions, the washed cells of *E. coli* grown aerobically on glucose medium reveal the ability to synthesize α-ketoglutaric acid from lactic acid, in a good yield, which would be the major end-product of its anaerobic fermentation.

The production of α-ketoglutaric acid from lactic acid containing three carbon atoms would necessarily involve a synthesis of the carbon chain as in the case of pyruvic acid already mentioned. Thus, Table VIII shows that α-ketoglutaric acid is obtained in a yield of about 0.3 mole from two moles of lactic acid. Subsequently, experiments on growing cultures were performed with the media containing glucose or glucose plus lactic acid as a source of carbon besides such mineral matters as those already mentioned [0.1% $(NH_4)_2HPO_4$, 0.1% $(NH_4)_2SO_4$].

The media were inoculated with *E. coli* and incubated on the shaker at 30°C for 6–10 days. Lactic

TABLE VII

PRODUCTION OF α-KETOGLUTARIC ACID FROM PYRUVIC ACID BY CELL-FREE EXTRACTS OF *E. COLI* GROWN AEROBICALLY ON GLUCOSE MEDIUM

Cell-free ext. (ml)	(Cells used g.)	$MgSO_4$ (μM)	Initial pyruvic acid (mM)	Pyruvic acid consumed (mM)	α-Ketoglutaric acid produced (mM)
15	0.5	40	1.600	0.382	0.047
15	1.0	50	1.600	1.600	0.165

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[4] T. Sasakawa et al., *Symposia on Enzyme Chemistry*, **10**, 103 (1954).

TABLE VIII

PRODUCTION OF α-KETOGLUTARIC ACID FROM LACTIC ACID BY WASHED CELLS OF *E. COLI* GROWN AEROBICALLY ON GLUCOSE MEDIUM

Bacterial cells (mg)	Phosphate buffer (mM)	$MgSO_4$ (μM)	Initial L-lactic acid (mM)	Total Volume (ml)	L-Lactic acid consumed (mM)	α-Ketoglutaric acid produced (Molar yield)
71	1	60	2.335	20	1.340	0.172
354	10	300	11.675	100	7.700	0.152

TABLE IX

PRODUCTION OF α-KETOGLUTARIC ACID FROM GLUCOSE OR GLUCOSE PLUS LACTIC ACID BY GROWING CULTURE OF *E. COLI*

Source of carbon					
	Ca-Lactate				
Conc. of glucose (%)	Conc. of free acid (%)	Optical form of free acid	Time of cultivation (days)	Consumption of glucose (%)	α-Ketoglutaric acid produced (mg/dl)
2.00	0		6	100.0	Trace
2.00	4.00	DL-	6	100.0	988.0
2.00	4.00	L-	6	100.0	877.0
4.00	0		10	100.0	1395.0
4.00	4.00	DL-	10	100.0	3294.0

TABLE X

INFLUENCE OF *o*-PHENANTHROLINE UPON OXIDATION OF LACTIC ACID BY WASHED CELLS OF *E. COLI* GROWN AEROBICALLY ON GLUCOSE MEDIUM

o-Phenanthroline added (μM/40 ml)	0	25
L-Lactic acid used (mM)	4.150	4.150
α-Ketoglutaric acid produced (mM)	0.500	Trace
Acetic acid produced (mM)	Trace	2.08

acid was again found to be an exceedingly potent precursor in the synthesizing reaction of α-ketoglutaric acid as will be seen in Table IX in which the amount of produced α-ketoglutaric acid was in such a high level as 3.294 g with 100 ml of a medium containing both 4% glucose and lactic acid as sources of carbon, while the yield was 1.395 g per 100 ml when 4% glucose was employed as the sole source of carbon.

Table X shows the effect of *o*-phenanthroline having the ability of forming a Fe-complex compound upon the oxidation of lactic acid by the washed cells of *E. coli* grown on 2% glucose medium. It will be seen that the production of α-ketoglutaric acid was extremely diminished, while, acetic acid increased when a reaction mixture (40 ml) containing 5 mM phosphate buffer (pH: 6.0), 100 μM $MgSO_4$, 175 mg bacterial cells, and 4.15 mM Na-L-lactate, was incubated on the shaker in the presence of *o*-phenanthroline (25 μM per 40 ml reaction mixture) at 30°C for 13 hours.

DISCUSSION

The formation of α-ketoglutaric acid from pyruvic acid may be represented in the following equation in which the synthesis of the carbon chain would necessarily be involved.

$$2\mathrm{CH_3\cdot COCOOH}+2\mathrm{O}\rightarrow \mathrm{HOOC\cdot CO\cdot (CH_2)_2COOH}+\mathrm{CO_2}+\mathrm{H_2O} \qquad [\mathrm{I}]$$

Under the authors' experimental conditions, α-ketoglutaric acid was obtained in such a good yield as 0.5–0.7 mole from two moles of pyruvic acid, that is, the yield amounted to 50–70% of pyruvic acid used. Furthermore, the experimental results with pyruvic acid, not containing high energy phosphate-bond (~Ph) in the molecule, will point out the propriety of the assumption that a major pathway of synthesizing α-ketoglutaric acid would not involve carbon dioxide fixation (oxaloacetic acid formation from pyruvate). On the other hand, phosphoenol pyruvic acid is known to be most effective upon carbon dioxide fixation in both animal and plant tissues5)6)7). As a further explanation for reaction 〔I〕, the following pathway would be possible:

$$2\ \text{Pyruvic acid} \xrightarrow{+O} \left.\begin{array}{l}\text{Acetyl-CoA}\\ \text{Pyruvic acid}\end{array}\right\} \xrightarrow[\text{condensation}]{+O} \begin{array}{l}\alpha\text{-Ketoglutaric acid}\\ +CO_2 \quad +H_2O\end{array} \qquad 〔II〕$$

(Pyruvate-acetate reaction)

The transformation of lactic acid into α-ketoglutaric acid was confirmed to proceed via pyruvic acid, based on the results that a minute trace of pyruvic acid could be detected during the oxidation of lactic acid.

From the ecological point of view, it is interesting to note that lactic acid, which is accumulated as the major product without being metabolized in the anaerobic fermentation by the bacteria of *coli-aerogenes*, is available as a source of carbon and moreover is converted in aerobic conditions into α-ketoglutaric acid, which is the precursor of glutamic acid, therefore the starting substance for the syntheses of various amino acids.

SUMMARY

1) The production of α-ketoglutaric acid by the washed cells of *coli-aerogenes* from glucose was observed to be inhibited in the presence of αα′-dipyridyl. Moreover, it was found that both αα′-dipyridyl and *o*-phenanthroline extremely inhibited the bacterial synthesis of α-ketoglutaric acid from pyruvate, while the consumption of pyruvic acid was not greatly affected and acetic acid or acetoine could be produced even in the presence of these inhibitors. It was thus concluded that inorganic iron would be a kind of cofactor of the enzyme system relating to the synthesis of α-ketoglutaric acid from pyruvate, since these inhibiting actions could be reduced by the addition of inorganic iron such as $FeSO_4$.

2) It was demonstrated that the enzyme preparations such as dried cells and cell-free extracts revealed the ability of synthesizing α-ketoglutaric acid from pyruvate.

3) Lactic acid was found to be a potent precursor in the production of α-ketoglutaric acid by either the washed cells or the growing culture, and the same inhibiting action was again observed by the reagents forming Fe-complex compound.

4) The most presumable mechanism for the synthesis of α-ketoglutaric acid from C_3-compound such as pyruvic and lactic acids was discussed.

The authors are grateful to Mr. T. Sugimori for kindly supplying the sample of optically active lactic acid.

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[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 357~363, 1957]

Fractionation of Cigarette Smoke Components and Some Low Boiling Point Nitrogenous Compounds (I)*

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The chemical components of cigarette smoke produced in constant volume continuous smoking by an artificial smoking device were systematically fractionated into basic, acidic, neutral, phenolic, carbonyl and mercuric chloride-precipitable compounds. From the low boiling basic fractions ammonia, methylamine and ethylamine were qualitatively identified by paper chromatography, and pyridine and nicotine were isolated and identified by elementary analyses, mixed examinations and infrared spectra. An unidentified substance having elementary analysis values of C, 29.40; H, 1.93; N, 22.40 as picrate (m.p. 250°C, dec.) was isolated.

In the 1930's and in the early 1940's, the chemical components of tobacco smoke, especially nicotine and carbon monoxide, were studied from the hygienic viewpoint by A. Wenusch, C. Pyriki and others. Thereafter the studies were for a while in a state of abeyance, but since the early 1950's a fair number of studies concerning the individual chemical components of cigarette smoke have been actively resumed1~11). Recently, several authors in the fields of medical science have studied the polynuclear hydrocarbons contained in tobacco smoke in connection with carcinogenic substances 12~23). The most recent available information on the chemical components of tobacco smoke is that of A.I. Kosak24), who reviewed a number of literatures and has briefly summarized over 80 individual components in his review. There are, however, still a considerable number of minor components on which further study should be made.

Regular- and king-size cigarettes being used, the transfer and deposition of nicotine in cigarette smoke and butt have been studied

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[*] Part IV of a series of papers entitled "Studies on Tobacco Smoke".

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2) D.A. Buyske, L.H. Owen, P. Wilder, Jr. and M.E. Hobbs, *Anal. Chem.*, **28**, 910 (1956).

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6) J.D. Mold and M.T. McRae, *Tobacco*, **144** (No. 10), 24 (1957).

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11) G.P. Touey, *ibid.*, **27**, 1788 (1955)

12) E.T. Alvord and S.Z. Cardon, *Brit. J. Cancer*, **10**, 498 (1956).

13) J. Bonnet et S. Neukomm, *Helv. Chim. Acta*, **39**, 1724 (1956).

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21) H. Lettré, A. Jahn und Ch. Hausbeck, *Angew. Chem.*, **68 Jg.**, 212 (1956).

22) D. Schmäl, U. Consbruch und H. Druckrey, *Arzneim.-Forsch.*, **4**, 71 (1954).

23) C. Seelkopf, *Zeitschr. f. Lebensm.-Unters. u. -Forsch.*, **100**, 218 (1955).

24) A.I. Kosak, *Experientia*, **X/2**, 69 (1954).

and reported already in the previous papers[25,26]. During the course of a series of studies on the physical and chemical properties of cigarette smoke it has become desirable to investigate the minor smoke components related to the aroma and taste of Japanese tobaccos. The present communication deals with the fractionation of cigarette smoke components which were produced in constant volume continuous smoking by an artificial smoking device, and with the isolations and identifications of some low boiling point nitrogenous compounds.

The fractionation was conducted in a manner of making the cigarette smoke absorb in a train of absorbing bottles containing ether, sulfuric acid, sodium hydroxide, 2,4-dinitrophenylhydrazine-hydrochloric acid, and mercuric chloride solutions, respectively, and in a procedure of functional grouping.

By paper chromatography employing some different solvent systems, ammonia, methylamine and ethylamine were qualitatively identified, and the existence of the other two spots of unidentified substances was indicated. Pyridine and nicotine were isolated and identified by elementary analyses, mixed examinations and infrared spectra. An unidentified substance having elementary analysis values of C, 29.40; H, 1.93; N, 22.40 as picrate was also isolated. Among these substances, the confirmation of ethylamine in cigarette smoke has only uncertainly been reported in a literature.

The isolations and identifications of the individual smoke components contained in the other fractions are now in progress.

EXPERIMENTAL

I. Sample Cigarettes and Smoking Conditions: The cigarettes employed in this study were uncased regular-size cigarettes of 70-mm in length, 26-mm in circumference, and were made of a 66.9-5.1-9.1-14.0-4.9 blend of Japanese Flue-cured, Indian Flue-cured, Japanese Native "Matsukawa", Japanese Native "Tôzan" and Indian Native tobacco leaves by a Japan Monopoly Corporation type (Chûken type) cigarette making machine. After conditioning in an atmosphere adjusted to a relative humidity of 60% at 25°C, cigarettes falling in the weight ranges of 1.125 g ±5% (filled cut-leaves 1.078 g per one cigarette) were stored in closed vessels at 25°C and used throughout the investigation. The moisture content of the cigarettes was 10.0%.

An artificial smoking device shown in Fig. 1 being used, 22,000 cigarettes were continuously smoked, four by four, in a constant smoking volume, 20 cc/sec. per one cigarette, which simulated the puff volume of 40 cc/2 sec. in human smoking. This constant smoking volume was maintained by adjusting it with a flow meter (B) throughout the experiments. When the burning zone of each cigarette reached to a pencil mark, initially 47 mm from the lighted end, the cigarette-butt of 23 mm in length was removed from the cigarette holder (A) and another cigarette was inserted to continue smoking.

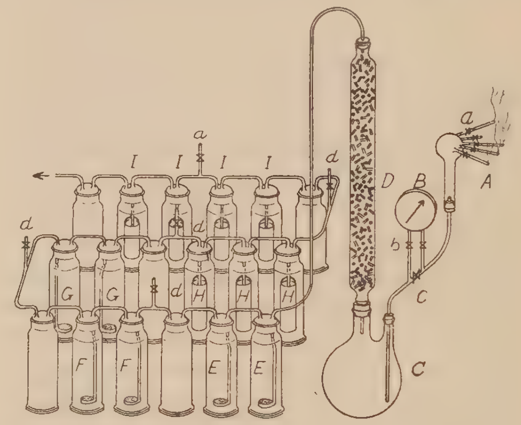

FIG. 1. Artificial Smoking Device and Smoke Collection Train.

A, cigarette holders; B, flow-meter; C, 1-liter flask; D, packed tower. Absorbing bottles: E, ether; F, 3% H_2SO_4 aq.; G, 1% NaOH aq.; H, 0.2% 2,4-dinitrophenylhydrazine-2N HCl aq.; I, 5% mercuric chloride aq.. Cocks: a, cigarette holder cocks (opened in case of smoking, except one cock); b, flow-meter cocks (closed in case of smoking); c, by-pass cock (opened in case of smoking); d, emergency cocks (closed in case of smoking).

II. Collection of Smoke Components: The cigarette smoke produced in this manner was collected in a smoke collection train consisting of a 1-liter flask

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[25] M. Izawa and Y. Kobashi, *J. Agr. Chem. Soc. Japan*, **29**, 754 (1955).

[26] Ibid., *ibid.*, **30**, 332 (1956).

with two necks (C), a tower packed with a large number of short glass tubes and beads (D) (42 mm diameter, 550 mm length), and thirteen 200-ml absorbing bottles containing ether (E), 3% sulfuric acid aq. (F), 1% sodium hydroxide aq. (G), 0.2% 2,4-dinitrophenylhydrazine-2 N hydrochloric acid aq. (H) and 5% mercuric chloride aq. solutions, respectively. E, F and G were absorbing bottles with glass-filters, and H and I were Milligan type ones. Five empty bottles were placed between each unit absorbing bottle in order to prevent the contamination with the neighboring solutions. The ether or aqueous solutions in the bottles were renewed on each occasion.

III. Fractionation of Smoke Components:

Smoke Components Collected in Ether: About 40 l of ethereal solution of cigarette smoke components was obtained after the smoking of 22,000 cigarettes. This ethereal solution was pulled at a room temperature through a collection train similar to the abovementioned one by passing nitrogen gas, and this treatment gave 5 l of concentrated ethereal smoke-solution, which was fractionated according to functional groups in a manner described in Table I.

Weak acidic solutions remaining in a set of cigrette holders (A), the flask with two necks (C) and the packed tower (D) were pooled their aqueous and ethereal washings. The combined solution was fractionated in the same procedure as described in Table

TABLE I

FRACTIONATION OF SMOKE COMPONENTS COLLECTED IN ETHER-ABSORBING BOTTLES (E)

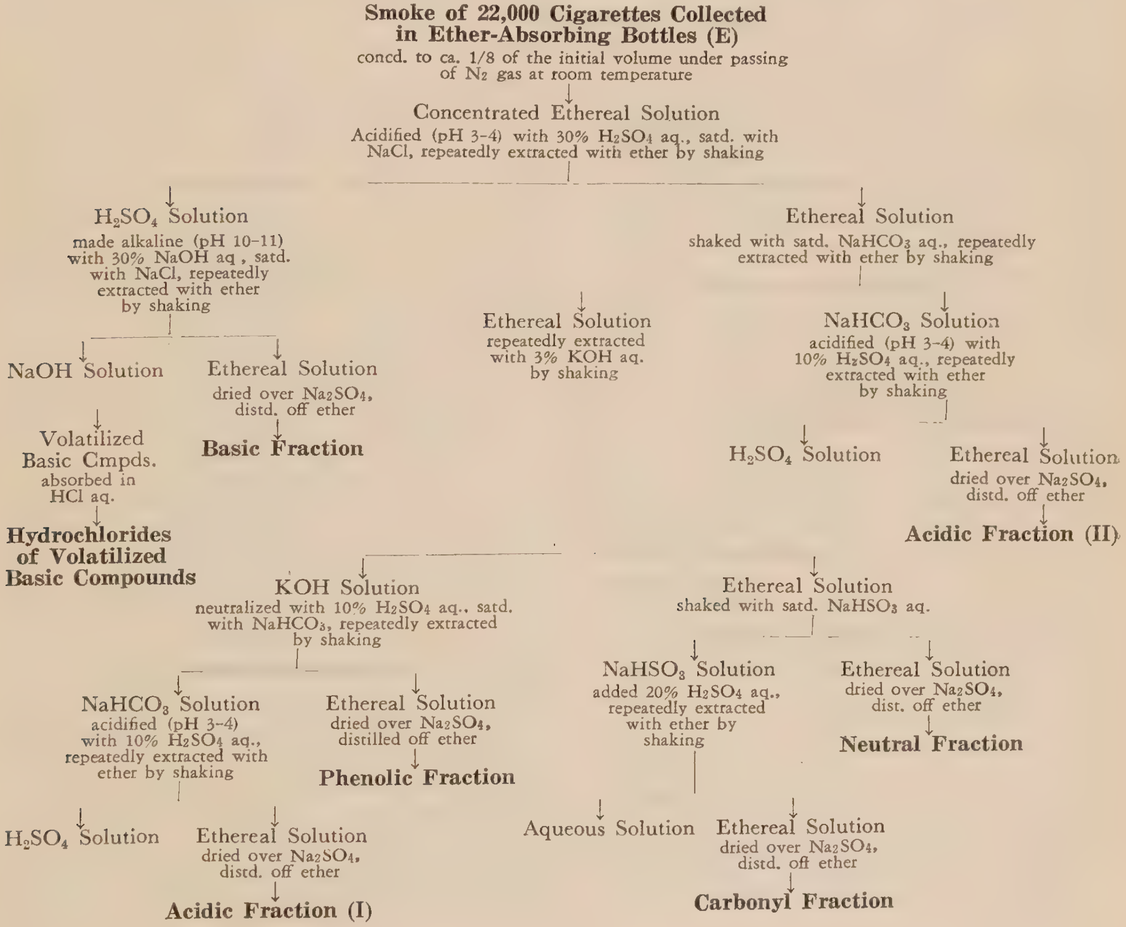
Smoke of 22,000 Cigarettes Collected in Ether-Absorbing Bottles (E)
concd. to ca. 1/8 of the initial volume under passing of N2 gas at room temperature
Concentrated Ethereal Solution
Acidified (pH 3–4) with 30% H2SO4 aq., satd. with NaCl, repeatedly extracted with ether by shaking
H2SO4 Solution
made alkaline (pH 10–11) with 30% NaOH aq., satd. with NaCl, repeatedly extracted with ether by shaking
NaOH Solution
Volatilized Basic Cmpds. absorbed in HCl aq.
Hydrochlorides of Volatilized Basic Compounds
Ethereal Solution
dried over Na2SO4, distd. off ether
Basic Fraction
Ethereal Solution
shaked with satd. NaHCO3 aq., repeatedly extracted with ether by shaking
Ethereal Solution
repeatedly extracted with 3% KOH aq. by shaking
NaHCO3 Solution
acidified (pH 3–4) with 10% H2SO4 aq., repeatedly extracted with ether by shaking
H2SO4 Solution
Ethereal Solution
dried over Na2SO4, distd. off ether
Acidic Fraction (II)
KOH Solution
neutralized with 10% H2SO4 aq., satd. with NaHCO3, repeatedly extracted by shaking
NaHCO3 Solution
acidified (pH 3–4) with 10% H2SO4 aq., repeatedly extracted with ether by shaking
H2SO4 Solution
Ethereal Solution
dried over Na2SO4, distd. off ether
Acidic Fraction (I)
Ethereal Solution
dried over Na2SO4, distilled off ether
Phenolic Fraction
Ethereal Solution
shaked with satd. NaHSO3 aq.
NaHSO3 Solution
added 20% H2SO4 aq., repeatedly extracted with ether by shaking
Aqueous Solution
Ethereal Solution
dried over Na2SO4, distd. off ether
Carbonyl Fraction
Ethereal Solution
dried over Na2SO4, dist. off ether
Neutral Fraction

TABLE II

PAPER CHROMATOGRAPHY OF LOW BOILING NITROGENOUS COMPOUNDS OF CIGARRTTE SMOKE (Hydrochlorides)

Chlorides of	*n*-butanol : acetic acid : water[27] (4 : 1 : 5)				*n*-butanol : ethanol : water[28] (4 : 1 : 1)					*tert*.-amylalcohol : buffer[29] (50 : 50)	
	Authentic	Specimen			Authentic	Specimen				Authentic	Specimen
		Ninhydrin	Iodine	BrCN		Ninhydrin	Iodine	BrCN	Nessler's		Nessler's
Ammonia	—	—	—	—	0.16	–	–	–	0.16LB	0.23	0.23LB
Methylamine	0.34	0.34PP(PRP)	–	–	0.21	0.22PP(PRP)	–	–	–	–	–
Ethylamine	0.44	0.43PP(PRP)	–	–	0.31	0.31PP(PRP)	–	–	–	–	–
Trimethylamine	0.44	–	–	–	0.26	–	–	–	–	–	–
Nicotine	0.59	–	–	–	0.66	–	–	–	–	–	–
Unidentified substance (1)	—	–	0.70LB	–	—	–	–	–	–	—	–
Unidentified substance (2)	—	–	0.47LB	0.47YO	—	–	0.31LB	0.31YO	–	—	–

Remarks: Filter paper—" Toyo " No. 50. Ascending method. 28–31°C. Buffer—Mixture of 0.2 M acetic acid aq. and 0.2 M Na-acetate aq. (9.5 ml: 90.5ml), pH 5.6. Ninhydrin—0.2% ninhydrin-butanol soln.. Colors enclosed with brackets are those obtained by 0.8% Cu-nitrate alcoholic soln.-spraying after the ninhydrin-butanol soln.-treatment. Iodine—1% iodine-95% ethanolic soln.. BrCN—After spraying with 1% *p*-amino benzoic acid-ethanolic soln., the paper was exposed to BrCN vapor for 15–20min.. Nessler's—Nessler's reagent. PP—Pale purple, PRP—Pale reddish purple, LB—Light brown, YO—Yellowish orange.

I. The individual fractions thus obtained were combined to the fractions described in Table I.

Smoke Components Collected in Sulfuric Acid and Sodium Hydroxide Solutions: The content of 3% sulfuric acid-absorbing bottles (F) in Fig. 1 was pooled the solution remaining in the sulfuric acid-absorbing bottles which had been used for collecting volatile basic compounds at the concentration of the above-mentioned ethereal smoke-solution. After making alkaline with a 30% sodium hydroxide aq., this combined solution was repeatedly extracted with ether. A great portion of ether in the ethereal extract was distilled off in vacuo, then the distillate below 50°C/20 mm. Hg was collected in a collection train containing 3% hydrochloric acid aq.. Evaporation to dryness of these hydrochloric acid solutions gave 0.058 g of hydrochlorides. The amount of the residual liquor in the distillation was 0.643 g as hydrochlorides.

An acidic fraction of 1.10 g was obtained by ether-extraction after acidifying the smoke-solution which was collected in the 1% sodium hydroxide-absorbing bottles (G) in Fig. 1. Another acidic fraction weighing 5.88 g was also gained from the sodium hydroxide-absorbers at the concentration of the above-mentioned ethereal smoke-solutions.

Smoke Components Collected in 2,4-Dinitrophenylhydrazine-Hydrochloric Acid Aqueous Solutions: Hydrazones of carbonyl compounds, which were precipitated in the 2,4-dinitrophenylhydrazine-absorbing bottles (H) at the smoking and similar ones at the concentration of the above-mentioned ethereal smoke-solutions, were filtered, washed with water, and dried. The weights of both hydrazones were 157.80 g and 37.80 g, respectively.

Smoke Components Collected in Mercuric Chloride Aqueous Solutions: The mercuric chloride salts precipitated in the mercuric chloride-absorbers (I) weighed 53.99 g, and those of the similar absorbers at the concentration of the ethereal smoke-solutions weighed 9.44 g.

IV. Qualitative Identification of Ammonia, Methylamine and Ethylamine by Paper Chromatography: A portion of the hydrochlorides of the basic fractions, having boiling points below 50°C/20 mm. Hg, which were described in the paragraph of "Smoke components collected in sulfuric acid and sodium hydroxide solutions", was paper-chromatographed for amines (Table II). In the paper chromatography employing some different solvent systems[27,28,29], ninhydrin reaction showed the presence of methylamine and ethylamine, and Nessler's reaction showed the existence of ammonia. An unidentified substance (1) was estimated as one of tertiary amines because of its coloration with iodine, and an unidentified substance (2) seemed to be a pyridine compound according to its cyanogen bromide coloration.

V. Identification of Nicotine: Three hundred mg of the hydrochlorides of basic fractions above 50°C/20 mm. Hg, described in the preceding paragraph, was taken in a small Houben-type distillation flask, made alkaline with a concentrated sodium hydroxide aq. and distilled into a saturated picric acid-alcoholic solution under heating by a mantle heater. A crude picrate of 0.4 g was obtained. Recrystallization with ethanol yielded a picrate melting at 223°C. No depression was observed in its mixed m.p. with an authentic nicotine picrate. Its infrared spectrum and elementary analysis agreed with the authentic nicotine picrate.

Found: C, 43.20; H, 3.44; N, 18.35
Calcd. for $C_{22}H_{20}N_8O_{14}$: C, 42.59; H, 3.25; N, 18.06

VI. Identification of Pyridine and Isolation of an Unidentified Substance: In a similar manner to the above-mentioned procedure, 1.62 g of the hydrochlorides of volatilized basic compounds shown in Table 1 was made alkaline and distilled under heating at about 200°C. After 0.25 g of the picrate was yielded, the wall of the distillation flask was covered with a bandage so as to obtain the picrates of bases having a somewhat higher boiling point, and other picrates of 0.95 g were obtained.

The former picrates (0.2 g) were repeatedly recrystallized with water until a pure crystal of m.p. 250°C (dec.) was obtained. The elementary analysis of the purified picrate showed the values of C, 29.40; H, 1.93; N, 22.40. Owing to the small amounts of the specimen, the identification of this compound was not accomplished.

After the picrates of bases having a somewhat higher boiling point (0.2 g) were recrystallized four times with ethanol, a picrate having m.p. 163–164°C was obtained. This picrate showed no depression of mixed m.p. with the authentic pyridine picrate (m.p. 164°C). Its infrared spectrum and values of elementary analysis agreed with an authentic pyridine picrate.

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[27] W. Dihlmann, *Biochem. Zeitschr.*, **325**, 295 (1954).

[28] Y. Obata and K. Matano, *J. Agr. Chem. Soc. Japan*, **26**, 184 (1952).

[29] T.C. Tso and R.N. Jeffrey, *Arch. Biochem. Biophys.*, **43**, 269 (1953).

TABLE III

YIELDS OF ERACTIONATED CIGARETTE SMOKE COMPONENTS PER 22,000 CIGARETTES

	Cigarette smoke components		
Fraction	Collected in ether absorbing bottles (E) in case of smoking (g)	Collected in the other absorbing bottles (F, G, H or I) in case of smoking (g)	Recovered at the concentration of ethereal smoke-soln. in ether-absorbing bottles (E) (g)
Basic	82.75(+1.62*)	0.70*	
Acidic	94.36	1.10	5.88
Neutral	247.82	0	0
Phenolic	88.21	0	0
Carbonyl	17.37	157.80***	37.80***
$HgCl_2$-pptable	0	53.99****	9.44****

Remarks: *, Hydrochloride of volatilized basic cmpds. in Table I; **, Hydrochlorides; ***, 2,4-Dinitrophenylhydrazones; ****, $HgCl_2$-salts; "No asteriks" indicates free cmpds.

Found: C, 43.38; H, 2.34; N, 18.44
Calcd. for $C_{11}H_8N_4O_7$: C, 42.86; H, 2.62; N, 18.18

DISCUSSION

The yields of each fraction obtained from the cigarette smoke-solutions of 22,000 cigarettes are summarized in Table III. The basic fraction possesses fishy, pyridine- and/or nicotine-like odors which give an irritant odor of tobacco tar to cigarette smoke. The acidic fraction bears a strong acetic acid-like odor, and its low boiling part exhales the sharp or sweat-like odor of low molecular fatty acids, on the other hand the presence of substances having ester-like and soy-sauce-like aromas is also estimated. The amount of neutral fraction is the most abundant among all of the fractions. This fraction seems to contain the most characteristic aromatic substances which impart a fragrant, pleasant and/or unpleasant odor to cigarette smoke in a very small amount. The phenolic fraction emits a sharp odor of phenol. In the carbonyl fraction, characteristic odors, e.g., like scrambled eggs or pickled radishes, were observed. The mercuric chloride-precipitable compounds have a green laver-like aroma and repulsive mercaptan-like odor. In spite of the unpleasant odor of mercaptan-like substances, the presence of a very small amount of these compounds seems to be important in manifesting and promoting other agreeable and fragrant smells of cigarette smoke. The aroma and taste of cigarette smoke seem to consist of the co-operative actions of many numbers of compounds, because a single substance manifesting just cigarette flavor itself is not to be found in these fractions.

In this study, from the low boiling point nitrogenous compound fractions below 200°C under the ordinary pressure, ammonia, methylamine, ethylamine, pyridine and nicotine have been identified. Among these substances, ammonia, pyridine and nicotine have been found to exist in tobacco smoke by many authors, and methylamine has been also confirmed by Yu. O. Gabel et al.30) and A.W. Koperina31). But the presence of ethylamine has only uncertainly been indicated by J.S. Osborne et al.7). Trimethylamine, reported already by Yu. O. Gabel et al.30), has not been confirmed in this experiment, but the further studies in progress will clarify its presence together with more other low boiling point nitrogenous compounds. That nicotine has been found in the low boiling fraction, is perhaps due to insufficient fractionation.

Acknowledgement. The authors wish to ex-

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30) Yu. O. Gabel and G.I. Kipriyanov, *Ukrain. Khem. Zhur.*, **4**, Tech. Pt., 45 (1929); *Chem. Abstr.*, **24**, 916 (1930).
31) A.W. Koperina, *Biochem. Zeitschr.*, **219**, 258 (1930).

press their sincere thanks to Prof. Yatarô Obata of Hokkaido University for his guidance and encouragement in the course of this work, and to Director T. Nakashima and Chief. I. Ônishi of this Research Institute for their encouragement and support, and also to Dr. T. Yabuta, Emeritus Prof. of University of Tokyo, for his interest and advice. They are also indebted to the Laboratory of Microanalysis, Department of Agricultural Chemistry, University of Tokyo, for the microanalyses, and to Miss. T. Fukaya for her assistance in carrying out the experiments.

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Some Neutral Components of Cigarette Smoke (I)*

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The neutral fraction of cigarette smoke components, produced in constant-volume continuous smoking by an artificial smoking device, was sub-fractionated into alcohols, acid-, alcohol- and phenol-components of esters, unsaponifiable matters and hydrocarbons. From these sub-fractions ethyl alcohol, benzyl alcohol, β-phenylethyl alcohol and ethyl acetate were first isolated as their crystalline derivatives and then identified by their melting points, infrared spectra and elementary analyses. It is worth to note that the presence of these compounds in tobacco smoke has not yet been reported in the literature.

In a previous communication[1], it has been reported that the basic, acidic, neutral, phenolic, carbonyl and mercuric chloride-precipitable compounds can be obtained by the fractionation of cigarette smoke components produced in constant-volume continuous smoking by an artificial smoking device (regular size cigarettes; weight-ranges of filled cut-leaves, 1.078 g±5%; moisture content, 10.0%; smoking-volume, 20 cc/sec.; length of butts, 23 mm), and that the presence of ammonia, methylamine, ethylamine, pyridine and nicotine was confirmed in the low-boiling basic fraction of cigarette smoke components. The present paper deals with the sub-fractionation of the neutral fraction, and also with the isolation and identification of individual neutral compounds.

The neutral fraction of cigarette smoke[1] was divided into the steam-distillable part and the residual part. The latter was divided into two parts—the alcohol-soluble and the alcohol-insoluble—by their solubilities in absolute alcohol.

The steam-distillable part was fractionally distilled under a reduced pressure so as to divide it into the low-boiling components (below 140°C/760 mm. Hg) and the high-boiling components (above 140°C/760 mm. Hg).

As qualitative analyses indicated the presence of alcohols, esters and hydrocarbons, both the components were sub-frationated, respectively. The oxygenated compounds, obtained after the removal of hydrocarbons from the high-boiling components by a liquid-chromatographic technique, and the low boiling components were sub-fractionated into alcohol components, acid-, alcohol- and phenol-components of esters, unsaponifiable matters and hrdrocarbons.

The alcohol components were allowed to couple with 3,5-dinitrobenzoyl chloride, then the resulting derivatives were liquid-chromatographed, and ethyl alcohol, benzyl alcohol and β-phenylethyl alcohol were identified. Concerning the ester components, ethyl alcohol was identified in a similar fashion, and acetic acid was also identified as its *p*-bromophenacyl ester. Thus, the presence of ethyl acetate was confirmed.

Attempts to identify the following compouds resulted in a failure owing to their minute quantities: (1) An alcohol (m.p. of 3,5-dinitrobenzoate, 52–55°C) from the low-boiling components, (2) An organic acid (m.p. 245–246°C) from the acid- components of the esters in the low-boiling components, (3) Three different non-volatile acids which were estimated by paper chromatography on the acid-components of the esters of the high-boiling components, (4) A phenolic compound as a component of the esters, which was detected by paper chromatography in the phenol-components of the esters in the high boiling-components, (5) *tert*-Alcohols, which did not react with phthalic anhydride, and sulfur compounds, contained in the unsaponifiable matters, which were detected by qualita-

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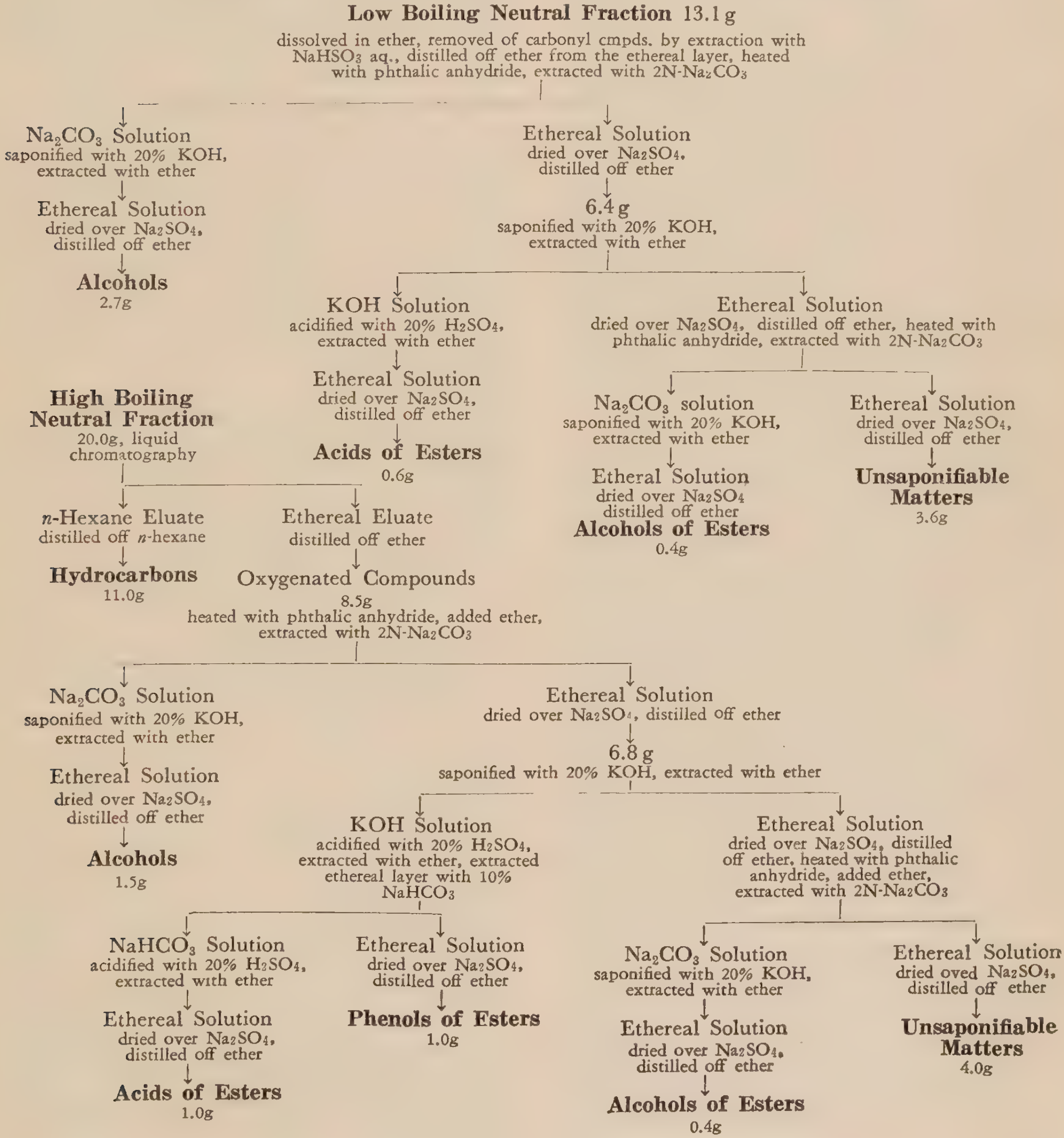
* Part V of a series of papers entitled "Studies on Tobacco Smoke"

[1] M. Izawa and Y. Kobashi, This Bulletin, **21**, 357 (1957).

TABLE I

SUB-FRACTIONATION OF THE NEUTRAL FRACTION OF CIGARETTE SMOKE


Low Boiling Neutral Fraction 13.1 g
dissolved in ether, removed of carbonyl cmpds. by extraction with NaHSO₃ aq., distilled off ether from the ethereal layer, heated with phthalic anhydride, extracted with 2N-Na₂CO₃
Na₂CO₃ Solution
saponified with 20% KOH, extracted with ether
Ethereal Solution
dried over Na₂SO₄, distilled off ether
Alcohols
2.7g
Ethereal Solution
dried over Na₂SO₄, distilled off ether
6.4 g
saponified with 20% KOH, extracted with ether
KOH Solution
acidified with 20% H₂SO₄, extracted with ether
Ethereal Solution
dried over Na₂SO₄, distilled off ether
Acids of Esters
0.6g
Ethereal Solution
dried over Na₂SO₄, distilled off ether, heated with phthalic anhydride, extracted with 2N-Na₂CO₃
Na₂CO₃ solution
saponified with 20% KOH, extracted with ether
Etheral Solution
dried over Na₂SO₄ distilled off ether
Alcohols of Esters
0.4g
Ethereal Solution
dried over Na₂SO₄, distilled off ether
Unsaponifiable Matters
3.6g
High Boiling Neutral Fraction
20.0g, liquid chromatography
n-Hexane Eluate
distilled off *n*-hexane
Hydrocarbons
11.0g
Ethereal Eluate
distilled off ether
Oxygenated Compounds
8.5g
heated with phthalic anhydride, added ether, extracted with 2N-Na₂CO₃
Na₂CO₃ Solution
saponified with 20% KOH, extracted with ether
Ethereal Solution
dried over Na₂SO₄, distilled off ether
Alcohols
1.5g
Ethereal Solution
dried over Na₂SO₄, distilled off ether
6.8 g
saponified with 20% KOH, extracted with ether
KOH Solution
acidified with 20% H₂SO₄, extracted with ether, extracted ethereal layer with 10% NaHCO₃
NaHCO₃ Solution
acidified with 20% H₂SO₄, extracted with ether
Ethereal Solution
dried over Na₂SO₄, distilled off ether
Acids of Esters
1.0g
Ethereal Solution
dried over Na₂SO₄, distilled off ether
Phenols of Esters
1.0g
Ethereal Solution
dried over Na₂SO₄, distilled off ether, heated with phthalic anhydride, added ether, extracted with 2N-Na₂CO₃
Na₂CO₃ Solution
saponified with 20% KOH, extracted with ether
Ethereal Solution
dried over Na₂SO₄, distilled off ether
Alcohols of Esters
0.4g
Ethereal Solution
dried oved Na₂SO₄, distilled off ether
Unsaponifiable Matters
4.0g

tive analyses, (6) A paraffin (m.p. 61–65°C) from the alcohol-insoluble part.

EXPERIMENTAL

I. Sub-Fractionation of the Neutral Fraction:

The neutral fraction of cigarette smoke components, 118.0 g, was steam-distilled in order to divide this fraction into a steam-distillable part, 34.5 g, and a residual part, 83.0 g. The latter was then divided into an alcohol-soluble part, 77.5 g, and an alcohol-insoluble part, 5.5 g.

The steam-distillable part was subjected to fractional distillation under a reduced pressure of 15 mm. Hg, and divided into two fractions according to the boiling point i.e., 13.1 g of the low-boiling components (b.p. below 140°C/760 mm. Hg) and 20.0 g of the high-boiling components (b.p. above 140°C/760 mm. Hg). Though the fractional-distillation was not so accurate, its purpose was complete enough to capture low boiling neutral compounds at the first-stage of the sub-fractionation, because at the subsequent stage it was feared that these compounds might be missed in distilling off *n*-hexane (b.p. 68–70°C) from the eluates obtained by liquid-chromatography for the separation of hydrocarbons and oxygenated compounds.

The low-boiling and high-boiling components were both sub-fractionated into individual neutral compounds as described in Table I.

The separation of alcohol components was carried out in the following manner. As the sodium nitroprusside reaction and fuchsin-sulfurous acid reaction showed the presence of an appreciable quantity of carbonyl compounds in the low-boiling components, 13.1 g of the low-boiling components was dissolved in ether, extracted with a saturated sodium hydrogen sulfite aq. to remove the carbonyl compounds. Ether was distilled off from the ethereal layer, and 5 g of phthalic anhydride was added; the mixture was then heated at the temperature of 130°C, on an oil bath for four hours. The reaction mixture was cooled, dissolved in ether, and shaked with 2N-sodium carbonate aq. to extract phthalic acid-esters and the unreacted reagent from the ethereal layer. Then, the extract was treated as shown in Table I.

In the case of alcohols, in the high boiling components a mixture of oxygenated compounds, 8.5 g, which had been separated liquid-chromatographically from 20.0 g of the high boiling components, was subjected to a reaction with 7.0 g of phthalic anhydride at 120°C for 3 hours, and then treated as in the foregoing experiments. The alcohol-components of the esters were also treated in a similar manner.

The liquid-chromatographic procedures employed for the separation of hydrocarbons and oxygenated compounds in the high-boiling components, were essentially the same as those described by J.G. Kirchner et al.[2]. A column (3×40 cm) was packed with 40 g of silicic acid which had been dried at 120°C for 3 hours, subsequently slurried with about 120 ml of *n*-hexane (b. p. 68–70°C). The excess *n*-hexane had no sooner been removed under a nitrogen pressure of about 250 mm. Hg, than 20 g of the high boiling components was carefully poured onto the top of the column, and developed with *n*-hexane under a nitrogen pressure of about 250 mm. Hg. Before eluate samples showed a negative color-reaction with a mixture of concentrated sulfuric acid and concentrated nitric acid (19:1 by vol.), 2 liters of *n*-hexane eluate containing hydrocarbons had been collected. After the *n*-hexane had been replaced by anhydrous ether, 1.5 liters of the eluate containing oxygenated compounds was obtained. The quantities of the hydrocarbons and oxygenated compounds after distilling off the solvents, were 11.0 g and 8.5 g, respectively.

The yields of each sub-fractionated components are shown in Table II. The figure in the quantities of these components in Table II must only be taken as a lower limit, inasmuch as considerable losses were sustained in the sub-fractionation processes.

Measurement of the saponification value of the mixture of components remaining after removal of alcohol

TABLE II

YIELDS OF SUB-FRACTIONATED COMPONENTS OF THE NEUTRAL FRACTION OF THE SUB-FRACTIONATED COMPONENT

	Yield (g)	Yield (%)
Neutral fraction	118.0	100.0
Steam-distillable part	34.5	29.2
Alcohols	4.2	3.6
Acids of esters	1.6	1.4
Alcohols of esters	0.8	0.7
Phenols of esters	1.0	0.9
Unsaponifiable-matters	7.6	6.4
Hydrocarbons	11.0	9.3
Steam-distillation residue	83.0	70.3
Alcohol-soluble part	77.5	65.7
Alcohol-insoluble part	5.5	4.7

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[2] J.G. Kirchner and J.M. Miller, *Ind. Eng. Chem.*, **44**, 318 (1952).

components from the oxygenated compounds, showed a value of 44.88.

II. Isolation and Identification of Alcohol-Components:

Isolation and Identification of Ethyl Alcohol: A portion (0.7 g) of the alcohol components of the low-boiling components was dissolved in 2 ml of anhydrous pyridine, and allowed to stand over night after adding 3.0 g of 3,5-dinitrobenzoyl choride. Then, the reaction mixture was poured into small quantities of ether, followed by successive washing with diluted hydrochloric acid, diluted sodium hydroxide and water. According to the procedures of J.K. Kirchner et al.[3] and I. Ônishi et al.[4], a very small amount of the ethereal solution of the reaction mixture was chromatographed with *n*-hexane containing ethyl acetate by 15% on a "chromatostrip", which had been prepared by spreading a paste of coagulated mixture of silicic acid (10 g), Japanese pharmacopoeial potato starch (0.6 g) and water (30 ml) on a glass strip; two colored spots were detected on the chromatogram by spraying a 0.5% α-naphthylamine-alcoholic solution according to the manner of R.G. Rice et al.[5]. Accordingly, for the isolation of these two different 3,5-dinitrobenzoates, the above-mentioned ethereal-solution was subjected to liquid-chromatography following the technique described by J.W. White, Jr., et al.[6]. The ethereal solution was mixed with a mixture of silicic acid and celite (2 : 1 by wt.) (ca. 5 g), distilled off ether, placed on the top of a column (2×15 cm) packed with a mixture of silicic acid and celite (2 : 1 by wt.) containing a very small quantity of rhodamine 6 G, and finally developed with a 5% solution of ether in *n*-hexane under a nitrogen pressure of 250 mm. Hg. After the 3,5-dinitrobenzoate, visible as two dark brown bands on a yellow fluorescent background under ultra violet irradiation, were eluted, eluates of the two bands were separately distilled off the solvents, and gave 2 mg of a white-needle crystal (m.p. 52–55°C) from the first eluate and 300 mg of a white-needle crystal (m.p. 93–94°C) from the second one after successive recrystallizations with *n*-hexane. The former could not be identified owing to its minute amount. The latter showed no depression of mixed m.p. with 3,5-dinitrobenzoate of an authentic ethyl alcohol (m.p. 94°C). Its infrared spectrum (by a Perkin-Elmer Model-21) and values of elementary analysis were identical with those of the authentic sample.

Found: C, 45.38; H, 3.07; N, 11.20
Calcd. for $C_9H_8N_2O_6$: C, 45.01; H, 3.36; N, 11.66

Isolation and Identification of Benzyl Alcohol and β-Phenylethyl Alcohol: The alcohol-components of the high-boiling components (1.5 g) was dissolved in 10 ml of anhydrous pyridine, added 5 g of 3,5-dinitrobenzoyl chloride, and heated at 100°C on a water bath for 1 hour. After cooling, the resulting mixture was successively washed with diluted hydrochloric acid, diluted sodium hydroxide and water. A very small quantity of the sample was used, only two spots were detected on a "chromatostrip"[3,4] by color reaction with α-naphthylamine[5]. Then, the specimen was divided into half and half, each half was liquid-chromatographed in the same manner[6] as described in the preceding paragraph, respectively. Two zones appeared in the column after development, and a white needle-crystal (270 mg, m.p. 93–94°C) was obtained from the eluate of the lower zone after recrystallizations with *n*-hexane. The crystal was characterized as 3,5-dinitrobenzoate of ethyl alcohol, m.p. 94°C, undepressed by admixture with an authentic sample. The infrared spectrum of the obtained crystal and that of the authentic one were identical. These facts verified the presence of ethyl alcohol in the high-boiling components. A crude white-crystal (40 mg, m.p. 73–91°C), which had been obtained from the eluate of the upper zone, was liquid-chromatographed once more. From the eluate of the lower band in this chromatography, a needle-crystal (9 mg, m.p. 111–113°C) was obtained, and another feather-like crystal (20 mg, m.p. 107–108°C) yielded after evaporation of the eluate of the upper band. The m.p. of the former crystal was not depressed by admixture with 3,5-dinitrobenzoate of an authentic benzyl alcohol (m.p. 113°C). The two crystals had identical infrared spectra and elementary analyses.

Found: C, 55.73; H, 3.34; N, 9.70
Calcd. for $C_{14}H_{10}N_2O_6$: C, 55.63; H, 3.34; N, 9.27

The latter crystal was shown to be a 3,5-dinitrobenzoate of β-phenylethyl alcohol by elementary analyses and infrared spectra, and by its mixed confirmation with an authentic sample (m.p. 108°C).

Found: C, 56.96; H, 3.71; N, 8.74
Calcd. for $C_{15}H_{12}N_2O_6$: C, 56.96; H, 3.83; N, 8.86

III. Isolation and Identification of Esters:

Isolation and Identification of Acetic Acid from

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[3] J.G. Kirchner, J.M. Miller and G.J. Keller, *Anal. Chem.*, **23**, 420 (1951).

[4] I. Ônishi, H. Tomita and T. Fukuzumi, This Bulletin, **20**, 61 (1956).

[5] R.G. Rice, G.J. Keller and J.G. Kirchner, *Anal. Chem.*, **23**, 194 (1951).

[6] J.W. White, Jr. and E.C. Dryden, *ibid.*, **20**, 853 (1948).

Acid-Components of Esters: The acid-components of the esters of both low- and high-boiling components were divided into volatile and non-volatile acids by steam-distillation, respectively, and the following paper chromatography⁷⁾ was worked up on the volatile acids. After spot positions on a filter paper sheet were dampened with diethylamine, a portion of the ethereal solution of the volatile acids of the low-boiling components was spotted on the spot positions, developed with *n*-butanol-water-diethylamine (100:15:1 by vol.) by an ascending technique. Spraying with an citrtc acid-acidic alcoholic solution of 0.04% bromcresol green (pH 5.5), gave a chromatogram showing one spot equivalent to acetic acid. Then, *p*-bromophenacyl esters were prepared from the volatile acids of the low-boiling components, and 180 mg of a white scaly crystal having m.p. 83–85°C, unchanged by admixture with a *p*-bromophenacyl ester of an authentic acetic acid (m.p. 84°C), was obtained. The infrared spectra and elementary analyses of the two substance were identical.

Found: C, 46.93; H, 3.84
Calcd. for $C_{10}H_9O_3Br$: C, 46.72; H, 3.52

The volatile acids of the high boiling components were treated in a similar manner as described above, and acetic acid was likewise identified by mixed examination and infrared spectra.

Non-volatile Acids of Acid-Components of Esters: A pale yellow crystal (15 mg, m.p. 150–175°C) was obtained from the non-volatile acids of the esters in the low-boiling components, and recrystallized repeatedly with acetone until it yielded a very small quantity of crystal (m.p. 245–246°C), which was developed, by a descending technique, on a filter paper sheet with a mixture of 100% water-saturated isobutanol and isobutanol (90 : 10 by vol.) containing formic acid in the proportion of 5%, and sprayed with a 0.04% bromcresol green-95% alcoholic solution (pH 8.0)⁸⁾. The crystal, however, gave only an indistinct spot on the chromatogram, and was used up in the repeated experiments. Attempts to identify this compound were unsuccessful.

The non-volatile acids of the esters in the high-boiling components gave three spots having R_F values of 0.73, 0.84 and 0.93 by a similar chromatography as described in the foregoing experiment. It was estimated that the acid having a R_F value of 0.73 corresponds to succinic acid and the acid having a R_F value of 0.84 is equivalent to adipic acid, this confirmation, however, could not be carried out owing to their minute amounts.

Isolation and Identification of Ethyl Alcohol from Alcohol-Components of Esters and Confirmation of Ethyl Acetate: From the alcohol-components of the esters of the low-boiling components, 3,5-dinitrobenzoates were prepared. The derivatives were chromatographed by a "chromatostrip" procedure³⁾⁴⁾, and one spot was observed on the chromatogram. Accordingly, the derivatives were recrystallized from petroleum ether (b.p. 40–70°C), and a white needle-crystal (15 mg, m.p. 93–94°C) was obtained. This crystal was proved to be a 3,5-dinitrobenzoate of ethyl alcohol after mixed examination with an authentic sample (m.p. 94°C), infrared spectra and elementary analyses.

Found: C, 45.53; H, 2.93; N, 11.12
Cald. for $C_9H_8N_2O_6$: C, 45.01; H, 3.36; N, 11.66

Ethyl alcohol was likewise identified in alcohol-components of the esters of the high-boiling neutral components in the same procedures as described above.

In the foregoing paragraph it was proved that a major portion of the acid-components of the esters of the low-boiling components is composed of acetic acid. This time, a large percentage of their alcohol-components was proved to be ethyl alcohol. Therefore, the presence of ethyl acetate is positive. It was similarly confirmed that ethyl acetate is present in the high-boiling components.

Phenol-Components of Esters: Phenylazo-dye derivatives were prepared from the phenol-components of the esters of the high-boiling components, and paper-chromatographed by a one-dimensional descending technique with a solution of *sec*-butanol-2% sodium carbonate aq. (1 : 1) according to the method essentially identical with that described by Wen-Hua Chang et al.⁹⁾. Besides the spots of residual phenolic substances, a spot of a R_F value of 0.71, which had been scarcely observed on a chromatogram before saponification, appeared. This showed that a phenolic compound, of which experiments are now in progress, is present as one of the hydroxy-components of the esters.

IV. Unsaponifiable Matters, Hydrocarbons and Steam-Distillation Residue:

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7) A.R. Jones, E.J. Dowling and W.J. Skraba: *Anal. Chem.*, **25**, 394 (1953).

8) Y. Kobashi, *Ref. Notes. Centr. Res. Inst., Japan Monop. Copr.*, No. **53**, 53 (1951); E. Wada and Y. Kobashi. *J. Agr. Chem. Soc. Japan*, **27**, 561 (1953).

9) Wen-Hua Chang, R.L. Hossfeld and Wm. M. Sandstrom, *J. Am. Chem. Soc.*, **74**, 5766 (1952).

Table III
Fractional Distillation of Hydrocarbons

Fraction No.	B.p. (°C/mm.Hg)	Bath-Temp. (°C)	Yield (g)
1	49– 55/7	170–190	1.71
2	55– 57/7	190–200	0.89
3	57– 65/7	200–215	0.46
4	64– 70/6	215–220	2.05
5	80–100/6	220–225	0.53
6	100–110/6	225–230	1.37
7	110–115/6	230–235	1.03
8	115–120/6	235–240	1.00
Residue	—	—	1.45

In the unsaponifiable matters of both low- and high-boiling components, *tert*-alcohols, not reacted with phthalic anhydride, were qualitatively detected, and the presence of sulfur compounds was also detected.

The hydrocarbons (11.0 g) were fractionally distilled under a reduced pressure (Table III). Their quantities were comparatively large in the fractions of 64–70°C/6 mm. Hg, 49–55°C/7 mm. Hg, 100–110°C/6 mm. Hg and 115–120°C/6 mm. Hg. The preparations of picrates from these fractions resulted in a failure.

From the alcohol-insoluble part of the steam-distillation residue, a paraffin, melting at 61–65°C, was obtained.

Further experiments on these components are now in progress.

DISCUSSION

As reported in a previous paper1), the quantities of the neutral fraction are the most abundant among all fractions of cigarette smoke, and the presence of many aromatic substances, which give sweet smells to cigarette smoke, is estimated. On the other hand, the presence of substances having unpleasant odors is also suspected. Among those contained in the neutral fraction, the unsaponifiable matters emit the sharpest odors; especially, the matters obtained from the low-boiling components bear the most characteristic odors. The steam-distillable part, accounting for about 30% of the neutral fraction, is the most fragrant part, from which ethyl alcohol, benzyl alcohol, β-phenylethyl alcohol and ethyl acetate have been isolated and identified. The presence of these compounds in tobacco smoke has not yet been reported in the literature. Ethyl alcohol has been found in leaf tobaccos by C. Neuberg et al.10) and in the essential oils of leaf tobaccos by I. Ônishi et al.4). Ethyl acetate in essential oils of leaf tobaccos has also been reported by I. Ônishi et al.4). Concerning tobacco smoke, only methyl alcohol, ethylene glycol and diethylene glycol have been reported up to the present11), but the latter two are expected to be present only in the smoke of tobaccos sprayed with such substances as humectants. Methanol was not found in the authors' experiments, so further studies must be made. Several authors have studied methanol together with carbon monoxide and cyanogen compounds in tobacco smoke from the hygienic point of view. Recently, J. S. Osborne et al.12) and R. J. Philippe et al.13) have also confirmed its presence in cigarette smoke by an infrared compensation absorption procedure and mass spectral study.

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10) C. Neuberg and M. Kobel, *Biochem. Zeitschr.*, **179**, 459 (1926).

11) A.I. Kosak, *Experientia*, **X/2**, 69 (1954).

12) J.S. Osborne, S. Adamek and M.E. Hobbs, *Anal. Chem.*, **28**, 211 (1956).

13) R.J. Philippe and M.E. Hobbs, *ibid.*, **28**, 2002 (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 370~378, 1957]

Studies on Cardiotonic Substances Produced by Microorganisms. (I)

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The active substances such as cardiotonics or toxics which were produced in the cultured broths or contained in the mycellia of microorganisms were examined by Ito's assay method for cardiotonica using the embryonic heart of Japanese killi-fish.

There were some strains obtained from actinomycetes whose cultured broths or methanol mycelial extracts showed these activities, but fewer from moulds, yeasts and bacteria.

There were not a few strains of actinomycetes to be found whose cultured broths were highly toxic to fishes and some of these broths showed strong hemoloytic activities, consequently, it was considered that there might be some cardiotonic substances effective on human heart and circulation which are produced in the cultured broths or contained in the mycellia of microorganisms. In order to examine the above mentioned possibility, 75 kinds of representative strains, selected out from moulds, bacteria, yeasts and actinomycetes, were cultured. The samples were prepared from these cultured broths and the methanol extracts of mycellia. Their effects on heart were tested by Ito's assay method using the embryonic heart of *Oryzias latipes* (Himedaka, Japanese Killifish) for cardiotonics, which can substitute the complicated treatment with exercised-hearts of dogs, cats or other mammalians. At first, 25 known antibiotic substances were assayed by the method, and though most of them were found to be not so effective on the embryonic heart, some of them were fairly effective up to the concentration of 10^{-8} g/ml. Next, the cultured broths of 75 different strains and the methanol extracts of their mycellia were also tested for this purpose. There were very few strains obtained from moulds, bacteria and yeasts whose cultured broths or methanol cell extracts showed any influence on the embryonic heart when applied in a concentration of 1:10 and 1:100. But in the cultured broths of some strains of actinomycetes influences acting such as tonics or toxics were found, while the media, itself, showed entirely no influence at the same concentration.

METHOD AND EXPERIMENT

Exp. 1.

The assay method of Ito employing fish embryo was one involving the screening of cardiotonic substances. The embryo of *Oryzias latipes* whose heart rate was as regular as 50–60 per 20 seconds at 28°C, was treated at least over 3 hours at room temperature with 1.0 ml of hyaluronidase solution prepared by dissolving 2000 units of hyaluronidase (Mochida Pharm. Co.) in a 0.75% Ringer solution. Each test-sample was diluted with the 0.75% Ringer solution for fish (NaCl 0.75 g, KCl 0.02 g, $(NH_4)_2CO_3$ 0.02 g in 100 ml redistilled water, pH 7.3). The treated egg was laid into sample solution of various concentrations in a hollow glass under a microscope (magnification: ×100) and was observed in a humidistatic thermostat. The heart beat-rate was counted, and intensity, the nature of heart-beat and features of circulation were observed at 28°C for about 3 hours. The samples which showed no influence, neither cardiotonic nor toxic, and also those which did not show any change of rate, intensity, enlargement of vein or promotion of blood circulation were marked for further experiment.

The result from the test conduced with the antibiotic substances is shown in table I., in which observations are designated as follows:

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TABLE I

Substance	Concentration	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Digitoxin	Embryonic heart of	↘B	↘B	↗B	↗	↗	↗	↗
	Oryzias latipes (after 24 hrs.)	(+)	(+)	(−)	(−)			
	Adult *Oryzias latipes*	+	±	−				
	Daphnia	+	+	+	−			
Antimycin A	Embryonic heart of	↘B	↘B	↘B	↘B	↘B	↘	↘
	Oryzias latipes (after 24 hrs.)	(+)	(+)	(+)	(+)	(−)	(−)	
	Adult *Oryzias latipes*	⧺	⧺	⧺	+	−		
	Daphnia	⧺	⧺	⧺	+	−		
Magnamycin	Embryonic heart of	↘↗B	−B	−B	−	−	−	−
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	+	−	−				
Aureomycin	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Erythromycin	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Trichomycin	Embryonic heart of	↗B	−B	−B	−B	±	−	
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)	(−)	(−)	
	Adult *Oryzias latipes*	⧺	+	−	−			
	Daphnia	⧺	⧺	+	−			
Polymixin B	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	+	−	−				
	Daphnia	⧺	+	−				
Sarkomycin	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Netropsin	Embryonic heart of	↘	↘	±	−	−	−	
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)	(−)	(−)	
	Adult *Oryzias latipes*	+	−	−				
	Daphnia	⧺	+	−				
Bacitracin	Embryonic heart of	±	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	(−)	(−)	(−)				
Rimocidin	Embryonic heart of	↗B	↗B	↗B	↗B	±B	−	
	Oryzias latipes (after 24 hrs.)	(+)	(−)	(−)	(−)	(−)	(−)	
	Adult *Oryzias latipes*	⧺	+	−				
	Daphnia	+	−	−				
Eurocidin	Embryonic heart of	↗B	−B	−B	−B	±	−	
	Oryzias latipes (after 24 hrs.)	(+)	(+)	(±)	(−)	(−)	(−)	
	Adult *Oryzias latipes*	⧺	+	−				
	Daphnia	+	+	−				

Substance	Concentration	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Viomycin	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Leucomycin	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Chloramphenicol	Embryonic heart of	↗	↗	↗	±	−	−	
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)	(−)	(−)	
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Actidion	Embryonic heart of	−B	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	+	−	−	−			
	Daphnia	++	++	++	+	−		
Actonomycin	Embryonic heart of	−B	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	++	++	+	−			
	Daphnia	++	++	++	+	−		
Penicillin	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Streptomycin-sulfate	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(−)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	−	−	−				
	Daphnia	−	−	−				
Aureothricin	Embryonic heart of	B	B	−	−			
	Oryzias latipes (after 24 hrs.)	(+)	(+)	(−)	(−)			
	Adult *Oryzias latipes*	+	±	−				
	Daphnia	++	+	−				
Calcinofillin	Embryonic heart of	−	−	−	−			
	Oryzias latipes (after 24 hrs.)	(±)	(−)	(−)	(−)			
	Adult *Oryzias latipes*	++	+	−				
	Daphnia	++	+	±	−			

+——Influence of the substance on the embryonic heart of *Oryzias latipes*, certain.
±——Influence, uncertain.
↗——Increase in heart-rate.
↘——Decrease in heart-rate.
$\overline{\nabla}$ $\underline{\triangle}$—Revival to the initial-count after a temporary decrease or increase.
〰——Count, unstable.
B——Atrioventricular block.
S——Heart-beat, strengthened.
W——Heart-beat, weakened.

(+)——Death after 24 hours' treatment.
(−)——Survival after 24 hours' treatment, and not influenced.
(±)——Death after over 24 hours' treatment or decay of beat after treatment of over 24 hours.

From the results two antibiotic substances, Rimocidin and Eurocidin, were found to be effective on the embryonic heart. They increased the heart-rate over 10% in a concentration range of 10^{-5} g/ml to 10^{-8} g/ml having to toxicity effect at the concentration of less than 10^{-5} g/ml.

Antimycin and Blastmycin showed toxicity in concentrations as low as 10^{-8} g/ml and -10^{-10} g/ml, while other substances showed either little activity or toxicity. Aureomycin, Erythromycin, Polymixin B, Sarkomycin, Bacitracin, Viomycin, Leucomycin, Penicilline and Streptomycin sulfate showed no influence at concentrations of 10^{-4} g/ml, -10^{-7} g/ml on the heart.

In table I., observations with other aquatic organisms (*Dephnia magna* and adult *Oryzias latipes*) are also shown. There are to some extent parallel relationships between these series of observations with a few exceptions. The notation about *Daphnia magna* and *Oryzias latipes* is as follow:

Daphnia ....Complete death at room temperature within 1 hours. ....⧺
Complete death at room temperature within 3 days. ....+
Uncertain. ....±
Oryzias latipes ....Complete death at room temperature within 1 hour. ....⧺
Complete death at room temperature within 24 hours. ......+
Uncertain. ....±

Exp. 2.

In this experiment, adult *Oryzias latipes* was used for the preliminary screening, because much time can be spared than in the original egg method. The strains whose cultured broths or methanol cell extracts showed notable toxicity on fish were marked out for the next step of screening. The preparation of samples was as follows: 25 kinds of strains from moulds, 15 from yeasts, 10 from bacteria and 25 from actinomycetes were cultured for 3 days and 6 days (moulds), 2 days and 3 days (yeasts), 1 day and 3 days (bacteria), 2 days and 5 days (actinomycetes) in the following media:

Media for moulds

Glucose	100 g
Peptone	10 g
$NH_4H_2PO_4$	2 g
$MgSO_47H_2O$	0.5 g
$CaCl_2$	0.1 g
KNO_3	2 g
dist. water	1 l
pH 5.4–5.6	

For yeast

Glucose	30 g
$(NH_4)_2SO_4$	115 g
KH_2PO_4	1 g
$MgSO_4$	0.5 g
Yeast ext.	1 g
dist. water	1 l
pH 5.0–4.8	

For bacteria

Meat ext.	5 g
Peptone	5 g
NaCl	3 g
dist. water	1 l
pH 6.8–7.0	

For actinomycetes

Glucose	10 g
Meat ext.	5 g
Peptone	5 g
NaCl	3 g
dist. water	1 l
pH 7.4–7.6	

Amounts of 100 ml of cultured broths in 500-ml shaker flasks were filtered (moulds, actinomycetes) or centrifuged at 9000 r.p.m. (yeasts, bacteria). The mycellia were washed with sterilized 0.85% NaCl solution, ground with fine sillica (moulds, actinomyces) or with glass powder (yeasts, bacteria), and extracted twice, first, with 50 ml of methanol at pH 5.0–6.0 and then at pH 8.0. Two methanol extracts each of 50-ml in acid and in alkali, were mixed to 100 ml, and then neutralized. The methanol solution was evaporated to about 1/100 of the original volume and then diluted-back with distilled water to the original volume.

Each 5-ml of the filtered cultured broth and the cellular extracts were diluted with 45 ml of dechlorinated tap water in 9 inches Petri-dish and pH was adjusted to the contrast dechlorinated water. The 5 fishes (*Oryzias latipes*) were put into 50 ml of these testing solutions and observed at room temperature.

The notation in table II. is given as follows:

TABLE II

Mould	broth × 10		mycellia × 10	
	3 days	6 days	3 days	6 days
1. *Absidia archidio* AB–1	±	−	−	−
2. *Asp. awamori*	±	−	−	±
3. *Asp. oryzae* A	−	−	−	+
4. *Asp. candidus*	−	−	−	−
5. *Asp. niger*	+	−	−	−
6. *Asp. terreus*	−	−	±	−
7. *Cladosporium fluvum*	−	−	−	−
8. *Collybia vertipes*	±	−	−	−
9. *Dematium pullulaus*	−	−	−	−
10. *Fusarium lini*	−	−	+	+
11. *Rhizopus tamarii*	±	−	−	±

	broth × 10		mycellia × 10	
Mould	3 days	6 days	3 days	6 days
12. *Asp. fumigatus* 14–3	−	−	−	±
13. *Asp. nidulaus* 15–3	−	−	−	−
14. *Pen. roqueforti*	±	−	±	+
15. *Ilpex cousarus*	±	±	−	−
16. *Monascus anka*	−	−	−	−
17. *Pen. islandicum*	−	−	−	−
18. *Polyporus fruodsus*	−	−	−	−
19. *Rhizopus nigricans*	−	+	−	−
20. *Mucor pussilus*	−	−	−	−
21. *Rhizopus japonicus*	−	+	−	−
22. *Pen. chrysogenum*	±	−	−	−
23. *Rhizopus chinensis*	−	−	−	+
24. *Monascus purpureus*	−	−	−	−
25. *Sclertina arachidia*	−	−	−	−
Bacteria	1 day	3 days	1 day	3 days
1. *Ps. fluorescens* Klar Bact 2–8	−	−	+	−
2. *Aerob. aerogenes* Bact 1–1 ATCC 7256	−	−	−	−
3. *B. cereus* var. *mycoides* IFO 3039	−	−	−	+
4. *Proteus vulgaris* H–19 Denken Bact 2–4	−	−	−	−
5. *E. coli* Taki 2.	±	−	−	−
6. *Sarcina lutea* PCL 1001 Bact 2–1	−	−	−	−
7. *Erwinia carotvora* IFO 3308 Bact 5–6	−	−	−	−
8. *B. megatherium* NRRL-B-930 Bact 1–12	−	−	−	−
9. *B. subtilis* PCL 219 Bact 2–8	−	−	−	−
10. *Serratia marcescens* (Prod 3–1) NI 8069	−	−	−	−
Yeast	2 days	3 days	2 days	3 days
1. *Candida utilis* TH 9	+	+	±	−
2. *Endomyces fibriger* Zatsa 1–10	−	−	−	−
3. *Hansenula anomara* wil 7	+	+	±	−
4. *Kloeckera japonica* Zatsu 2–8	−	−	−	−
5. *Pichia* sp. chalon AY 2–6	+	+	−	−
6. *Rhodotorula gentinis* TH 6	+	−	−	−
7. *Saccharomyces cerevisiae* SH 4–7	+	+	−	−
8. *Rasse*-2 Shin 1–1	+	+	−	−
9. *Sac. rouxii* Zs-1	±	−	−	−
10. *Schizosacch. octosporus* SH S1–8	−	−	−	−
11. *Sacch. formosensis* Br 51 7–10	+	+	−	±
12. *Zygosacch.* sp. Zs-5 Soya yeast Higeta	−	−	−	±
13. *Torulopsis* sp. sherry yeast Shin 1–7	−	−	−	−
14. *Untergaerige Bier Hefe* Nunchen B 1–5	−	−	+	±
15. *Wine yeast* WH 5–2 OC 2	−	±	−	−
Actinomyces	2 days	5 days	2 days	5 days
1. *St. griseus* 3463	−	−	−	−
2. *St. lavendulae* 3340–8	+	+	+	+
3. NRRL B-546 St. antibioticus	+	+	+	+
4. *St. Hachijoensis* 2552	+	−	+	+
5. *St. albireticuli* (Eurocidin, Enteromycin, carbomycin)	−	−	+	±

6.	*St. thioluteus* 280–3	+	⧺	+	⧺
7.	*St.* KK 125 (Xanthomycin)	–	+	–	–
8.	*St.* 3317 (pikromycin) (Luteomycin)	–	–	–	±
9.	*St. rimosus* 2234	+	+	±	–
10.	*St. tanashiensis* (Luteomycin)	–	–	–	–
11.	*St. fradiae* 3535	–	⧺	–	+
12.	*St. virginiae*	–	–	–	–
13.	*St. vinaceus* B-1381	–	+	–	+
14.	*St. parvus* B-1255	–	–	–	+
15.	*St. albus* B-3381	–	⧺	–	⧺
16.	*St. olivaceus* PD 61	–	–	–	–
17.	*St.* O-2-B (Flavacid)	+	⧺	+	⧺
18.	*St.* 2A-343	⧺	⧺	+	+
19.	*St.* 2A 720	+	⧺	+	+
20.	*St.* 2A 1563	+	⧺	⧺	⧺
21.	*St.* S-206	+	–	–	+
22.	*St.* S–580	+	+	+	⧺
23.	*St.* S-710	⧺	⧺	+	+
24.	*St.* 6A 970	–	+	–	+
25.	*St. hygroscopicus* (Hygroscopin)	–	–	–	–

⧺——All fish in a Petri-dish, completely dead within 1 hour.

+——All fish in the dish, completely dead between 1 and 24 hours.

±——Some of the fish dead or influenced within 24 hours.

–——No fish influenced within 24 hours.

Results of the experiments conducted on 25 strains of moulds, 15 of yeast, 10 of bacteria and 25 of actinomyces are as follows:

	——on cultured broth		——on methanol extract of mycells	
	⧺ or +	±	⧺ or +	±
mould	3 strains	7	4	5
bacteria	0	1	2	0
yeasts	7	2	1	4
actinomyces	13	0	11	2

This indicates that there are many strains obtained from actinomyces whose cultured broth or methanol extract of mycellia have a marked toxicity on the fish (*Orz. lat.*), but fewer from mould or yeast, and the least from bacteria.

Exp. 3.

Strains of which cultured broths or methanol cell extracts showed high toxicity to adult *Oryzias latipes* were taken up for the next screening with the spawn, the result of which is shown in table III. The highly toxic sample solutions obtained by the culture of these strains were diluted by adding 99 parts and 999 parts of 0.75% Ringer's solution to 1 part of the broth or 1 part of cellular extract, and assayed for cardiotonic activities.

Table III. shows (notation is equal to that before) that there were more strains which produce cardiotonic or toxic substances on the embryonic heart of *Oryzias latipes* of actinomycetes, as compared with those of moulds, yeasts, and bacteria. Strains found to be effective at such a low concentration of 1 : 1000 were:

Streptomyces lavendulae,	cultured broth (2 days' and 5 days' culture).
S-580	cult. broth (2 days' and 5 days').
Streptomyces albus,	cult. broth (5 days').
Streptomyces fradiae,	cult. broth (5 days').
S-1563	methanol extract (3 days' and 5 days').
Echerchia coli.,	methanol extract (1 day).
Aspergillus niger,	cult. broth (3 days').
Penicillium roqueforti,	cult. broth (3 days'). methanol ext. (3 days').
Aspergillus terreus,	methanol ext. (3 days').

But the influences occurred much slower than that by digitalis cardiotonica. For example, the change of the count appeared 1 or 1.1/2 hours after treatment at 28°C.

Extraction of the effective substances was attempted employing several kinds of solvents for these influential fractions, and, for instance, in the case of *Streptomyces lavendulae*, the ethylacetate extract of the cultured broth showed the efficiency in both acid and neutral but not in alkaline condition. The isolation of the effective substance will be reported in another paper published later.

TABLE III

Actinomycetes	Culture & fraction	×100		×1000	
St. hachijoensis	2 days M	−	(−)	−	(−)
〃	5 days M	−	(−)	−	(−)
〃	2 days F	−	(−)	−	(−)
〃	5 days F	−	(−)	−	(−)
St. antibioticus NRRL B-546	2 days M	/↘	(−)	±	(−)
〃	5 days M	$\underline{/\searrow}$	(−)	−	(−)
〃	2 days F	$\underline{/\searrow}$	(−)	−	(−)
〃	5 days F	−	(−)	−	(−)
St. KK 125 (Xanthomycin)	2 days M	−	(−)	−	(−)
〃	5 days F	−	(−)	−	(−)
St. lavendulae	2 days M	−	(−)	−	(−)
〃	5 days M	↗	(−)	−	(−)
〃	2 days F	↗	(−)	↗	(−)
〃	5 days F	↗	(−)	↗	(−)
St. thioluteus	2 days M	$\underline{/\searrow}$	(−)	−	(−)
〃	5 days M	↘	(−)	−	(−)
〃	2 days F	−	(−)	−	(−)
〃	5 days F	−	(−)	−	(−)
St. S-710	2 days M	±	(−)	−	(−)
〃	5 days M	±	(−)	−	(−)
〃	2 days F	±	(−)	−	(−)
〃	5 days F	±	(−)	−	(−)
St. rimosus	2 days M	−	(−)	−	(−)
〃	5 days M	↗±	(−)	−	(−)
〃	2 days F	−	(−)	−	(−)
〃	5 days F	−	(−)	−	(−)
St. S-580	2 days M	−	(−)	−	(−)
〃	5 days M	−	(−)	−	(−)
〃	2 days F	↗	(−)	↗	(−)
〃	5 days F	↗	(−)	↗	(−)
St. fradiae	5 days M	↗	(−)	↗±	(−)
〃	5 days F	/\/\/↗	(−)	↗	(−)
St. vinaceus	5 days F	−	(−)	−	(−)
〃	5 days M	−	(−)	−	(−)
St. albus	5 days F	↗B	(−)	↗±	(−)
〃	5 days M	↗	(−)	↗±	(−)
St. S-206	2 days F	B	(−)	B	(−)
〃	2 days M	−	(−)	−	(−)
〃	5 days F	↘	(+)	±	(−)
〃	6 days M	−	(−)	−	(−)
St. parvus	5 days F	±	(−)	−	(−)
〃	5 days M	↗	(−)	±	(−)
St. 2A-343	2 days F	$\underline{/\searrow}$	(−)	−	(−)
〃	2 days M	−	(−)	−	(−)
〃	5 days F	\/\/\/↘	(−)	/↘	(−)
〃	5 days M	\/\/\/↘	(+)	↗	(−)
St. 2A-1563	2 days F	↗	(±)	−	(±)
〃	2 days M	↗	(+)	↗	(−)

F: filtrate M: methanol cellular extract.

St. 2A-1563	5 days F	↘	(−)	−	(−)
〃	5 days M	↗B	(+)	↗	(+)
St. S-3317	5 days F	B	(+)	−	(+)
〃	5 days M	±	(±)	±	(+)
Bacteria	Culture & fraction	×100		×1000	
Ps. fluorescens	1 day M	↗	(−)	−	(−)
E. coli	1 day M	↗	(+)	↗	(±)
〃	1 day F	∨±	(±)	−	(−)
B. cereus var. *mycoides*	3 days M	±	(−)	−	(−)
〃	3 days F	∧∧∧	(±)	−	(±)
Serratia marcessens	3 days M	−	(−)	↗	(±)
〃	3 days F	∧∧∧	(−)	−	(−)
Yeast	Culture & fraction	×100		×1000	
Candida utilis	3 days F	−	(+)	−	(−)
〃	2 days M	−	(+)	−	(−)
Hansenula anomara	2 days M	−	(−)	−	(−)
〃	3 days F	−	(−)	−	(−)
Pichia sp. charon	3 days F	−	(−)	−	(−)
〃	2 days F	−	(−)	−	(−)
Hansenula anomara	2 days F	±	(+)	−	(−)
Sac. cerevisiae	3 days F	−	(−)	−	(−)
〃	2 days F	↗	(+)	−	(−)
Rasse 2	3 days F	−	(−)	−	(−)
〃	2 days F	−	(+)	−	(−)
Rhodotorula glutinus	2 days F	−	(−)	−	(−)
Sac. formosensis	2 days F	±	(+)	−	(−)
〃	3 days F	↗	(+)	±	(−)
Untergarige Bierhefe	2 days F	↗	(±)	−	(−)
〃	3 days F	↗	(+)	±	(−)
Zygosac. Sp. 25-5	3 days M	−	(−)	−	(−)
Wine yeast oc-2	3 days F	±	(+)	−	(−)
Mould	Culture & fraction	×100		×1000	
Rhiz. nigricans	6 days F	±	(+)	−	(−)
Rhiz. jponicus	6 days F	−	(+)	−	(±)
Asp. oryzae	6 days M	−	(±)	−	(−)
Dematium pullulaus	6 days M	↗	(+)	−	(±)
Fusarium lini	6 days M	−	(+)	−	(±)
〃	3 days M	△	(+)	−	(±)
Pen. roqueforti	6 days M	−	(−)	−	(−)
〃	3 days M	↗	(+)	↗	(+)
〃	3 days F	↗	(+)	↗	(±)
Asp. awamori	6 days M	−	(−)	−	(−)
〃	3 days F	−	(+)	△	(−)
Rh. terreus	6 days M	↗	(−)	−	(−)
Rh. tamarii	6 days M	±	(−)	−	(−)
〃	3 days M	−	(+)	−	(−)
Rh. chinensis	6 days M	∇	(−)	−	(−)
Asp. niger	3 days F	↗	(+)	↗	(±)
Absidia archidia	3 days F	±	(+)	±	(−)
Pen. chrysogenum	3 days F	↗	(+)	±	(±)
Asp. terreus	3 days M	↗	(+)	↗±	(+)
Collybia vertipes	3 weeks F	−	(+)	−	(−)
〃	〃 M	±	(−)	−	(−)
Ilpex consorus	〃 F	−	(±)	−	(−)
〃	〃 M	−	(+)	−	(−)
Polyporus archidio	〃 F	−	(−)	−	(−)
〃	〃 M	−	(+)	−	(−)

SUMMARY AND DISCUSSION

The influences of substances contained in the cultured broths and in the mycellia of microorganisms on the embryonic heart of *Oryzias latipes* were examined under the assumption that there might be some cardiotonic substances produced by microorganisms. The experiments were carried out according to the assay method of Ito, using the embryonic heart of *Oryzias latipes*, which is a much easier and less expensive method using exercised mammalian hearts for cardiotonics. They were tested, at first, to the 25 known antibiotic substances and then to the cultures of 25 kinds of strains from moulds, 15 from yeasts, 10 from bacteria and 25 from actinomycetes.

Some of these antibiotic substances proved to be highly toxic while some were entirely ineffective and intoxic in the concentration range between 10^{-4} g/ml and 10^{-7} g/ml, but some were cardiotonic and not toxic at concentrations of less than 10^{-5} g/ml.

There were more strains highly toxic on adult *Oryzias latipes* of actinomyces in contrast to very few toxic strains of moulds, yeasts and bacteria. Since there were notable relationships among the results of the experiments on toxicity with the adult *Oryzias latipes*, *Daphnia* and the embryonic heart of *Oryzias latipes*, only the strains whose cultured broths showed toxicity to the adult fish were solely selected for the next screening test.

The result obtained was that there are several strains of actimomyces whose cultured broths or methanol cellular extracts are cardiotonic up to the dilution of × 1000.

The onset of the cardiotonic action was, however, slower compared with that of digitalis preparations.

The fact observed here was that there are some strains which produce cardiotonic substances effective on the embryonic heart and circulation of *Oryzias latipes*, and these activities are shown by comparatively many strains from actinomycetes but fewer from those of moulds, yeasts and bacteria.

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Studies on the Amylolytic System of Black-koji Molds

Part V. Debranching Activity of the Saccharogenic Amylase Fraction

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The saccharogenic amylase fraction was prepared from a black-koji amylase system, and its debranching activity was investigated. From its different attitude towards various chemical procedures, such as $(NH_4)_2SO_4$ fractionation, corn starch adsorption, and paper electrophoresis, it is suggested that two saccharogenic amylases one with and the other without debranching activity, may exist in the saccharogenic amylase fraction.

INTRODUCTION

Our previous study1) on the amylolysis caused by the black-koji mold amylase system showed that the saccharogenic amylase fraction completely hydrolyzes glycogen and glutinous rice starch to glucose, notwithstanding the fact that this fraction converts potato starch to an extent of about 90 per cent theoretical glucose with the remainder left as limit-dextrin stained purple by iodine. In the paper mentioned above, it was assumed that this fraction might contain a "debranching factor", that is, an agent capable of the breakdown of α-1,6-glucosidic linkages, which is more easily adsorbed on raw corn starch than the amylase itself, but it has not yet been made clear whether this factor represents isoamylase2), amylo-1,6-glucosidase3), R-enzyme4), limitdextrinase5), oligo-1,6-glucosidase6), or a sort of amylase with debranching activity.

In the present paper, evidence is presented, which supports the view that this "debranching factor" is a sort of amylase possessing debranching activity.

MATERIALS AND METHODS

Enzyme Preparation—Saccharogenic amylase fraction: The culture filtrate (10 l), prepared from the shaking culture of *Aspergillus awamori* (Kawachi strain) on a synthetic medium, was treated (pH 2.2, 60°C, 15 minutes) as described previously1) to destroy dextrinogenic amylase activity, and concentrated in vacuum at pH 3.8 (1 l). To the concentrate one-tenth of its volume of 1 per cent rivanol was added and after filtration, acid clay was added to the filtrate in order to adsorb the remaining rivanol. Then the precipitate, falling between 20 and 60 per cent ammonium sulfate, from the filtrate was dissolved into the water. The solution which was dialyzed against distilled water under toluene for two days, was used as a saccharogenic amylase fraction (250 ml).

Saccharogenic amylase fraction of another strain, *Aspergillus usamii*: This fraction was obtained by a 15-minute treatment of black-koji mold extract at 60°C, at pH, 7.8, then by concentration in vacuum, with further treatment of rivanol and fractionation with ammonium sulfate added thereto.

Preparations of Substrates—Beta-limit dextrin (β-LD) from glycogen was prepared by barley β-amylase after the usual method1). The preparation of soluble potato starch was obtained from a commercial source.

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TABLE I
$(NH_4)_2SO_4$ FRACTIONATION

Fraction	1		2		3	
Ammonium Sulfate g/100 ml	0–40		40–60		over 60	
Volume ml	52		55		16	
Substrate / Minutes	Soluble starch	β-LD	Soluble starch	β-LD	Soluble starch	β-LD
10	50.5	35.6	48.0	18.9	—	—
30	56.6	68.7	56.5	44.0	34.0	9.3
60	61.0	91.0	58.7	65.3	51.4	12.3
120	62.6	94.2	61.9	89.5	58.0	21.1

Figures show hydrolysis per cent.

Enzyme Activities—Saccharogenic activity and β-LD hydrolyzing activity were determined as described previously[1]. Debranching activity was determined by measuring the ratio of the β-LD hydrolyzing activity to the saccharogenic activity. In these experiments, the addition of the saccharogenic amylase free from debranching activity could not further promote the hydrolysis rate of β-LD by the debranching active amylase. The saccharogenic amylase activity, therefore, seems to exist excessively as compared with the debranching activity in any case.

Paper Electrophoresis—The experimental procedure employed for the paper electrophoresis was the same in principle as that described by Ando et al.[7].

RESULTS

Fractionation with Ammonium Sulfate—A quantity of 200-ml of the saccharogenic amylase fraction was fractionated with ammonium sulfate at pH 5.6. Saccharogenic and β-LD hydrolyzing activities were determined on each fraction. As it can be seen in Table I, the debranching active principle is precipitated rather in a lower concentration of ammonium sulfate. The debranching activity, however, was always accompanied by amylase activity.

Paper Electrophoresis—Paper electrophoresis was employed in order to separate the "debranching factor" from the saccharogenic amylase. A solutions of saccharogenic amylase fraction concentrated about 100-times was used as an enzyme solution. The paper electrophoresis was performed in the apparatus and method as described by Ando et al.[7].

A portion of 0.02 ml of enzyme solution was put on the starting line of strip (Toyo filter paper No. 50, 2×40 cm) which was wetted with 0.05 M phosphate buffer (pH 7.4), and subjected to a potential of 10 V per cm, 0.2 mA per cm for five hours in 0.05 M phosphate buffer (pH 7.4). After 5 hours, the filter paper (strip) was cut down into pieces of 0.5 or 1.0 cm wide, and each was immersed in 1.25-ml of water. Enzyme activities were determined on aqueous extract of each piece of strip. The reaction-system had the following composition.

1.6 per cent soluble starch solution	1 ml
1/10 M citrate buffer (pH 3.8)	1 ml
enzyme solution	0.5 ml
distilled water	2.5 ml

This reaction mixture was incubated at 30°C. In Table II, there is given the saccharogenic and β-LD hydrolyzing activities of the aqueous extract of each piece of strip. As it is shown, the debranching active principle moved to anode faster than the saccharogenic amylase did. Although the saccharogenic amylase which was deficient in debranching activity was obtainable (No. 2), the debranching activity without amylase could not be observed in any extract. Pieces No. 1 and No. 5 had no activity of either

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[7] T. Ando and Y. Ikeda, This Bulletin, **19**, 221 (1956).

TABLE II

THE SACCHAROGENIC AND β-LD HYDROLYZING ACTIVITIES OF EACH PIECE CUT OUT OF THE STRIP IN PAPER ELECTROPHORESIS

No.*	Hours / Substrates	16	54
		Hydrolysis per cent	
1	Soluble starch	0	0
	β-LD (glycogen)	0	0
2	Soluble starch	12.4	36.8
	β-LD (glycogen)	0	6.0
3	Soluble starch	56.0	61.4
	β-LD (glycogen)	22.7	41.6
4	Soluble starch	24.3	50.9
	β-LD (glycogen)	12.0	36.3
5	Soluble starch	0	0
	β-LD (glycogen)	0	0

* Number shows the distance (in cm) from the starting line to the anode.

amylase or β-LD hydrolyzing enzyme. Even when the extract of No. 5 was added to that of No. 2, no effect on both activities of the latter could be observed.

The Existence of the Debranching Activity in Another Black-koji Mold, *Aspergillus usamii*— The saccharogenic amylase fraction of this strain was obtained by the method mentioned above. As noted previously1), the adsorption treatment of the saccharogenic amylase fraction was carried out at pH 3.6. As shown in Fig. 1, the saccharogenic amylase fraction when subjected to adsorption lost its power to hydrolyze β-LD remarkably in comparison of about the same level of saccharogenic activity with the original enzyme solution. This trend coincided well in the case of *Aspergillus amamori* (Kawachi strain).

An experiment in the paper electrophoresis was made by using this fraction. The data are given in Table III. The debranching active principle, however, was found to move easily toward anode, but the separation of this activity from that of the amylase was a failure.

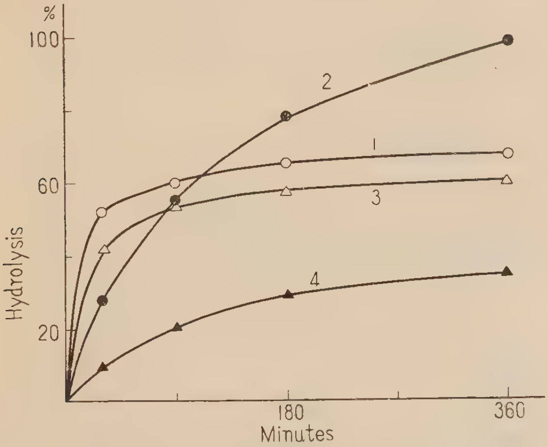


FIG. 1. Effect of Adsorption of Saccharogenic Amylase Fraction on Corn Starch in the Case of *Aspergillus usamii*.

Curve 1; non-treated saccharogenic amylase fraction on soluble starch.
Curve 2; non-treated saccharohenic amylase fraction on β-LD (glycogen).
Curve 3; treated saccharogenic amylase fraction on soluble starch.
Curve 4; treated sacchrogenic amylase fraction on β-LD (glycogen).

TABLE III

THE SACCHAROGENIC AND β-LD HYDROLYZING ACTIVITIES OF EACH PIECE OF THE STRIP IN PAPER ELECTROPHORESIS (*ASPERGILLUS USAMII*)

No.	Hours / Substrate	15	39	86
2	Soluble starch	21.0	49.6	53.8
	β-LD (glycogen)	4.2	7.9	17.4
3	Soluble starch	28.6	50.4	59.4
	β-LD (glycogen)	4.9	18.1	29.3
4	Soluble starch	26.6	52.4	60.6
	β-LD (glycogen)	10.2	23.8	45.0
5	Soluble starch	0	0	0
	β-LD (glycogen)	0	0	0

Purification and Properties of Saccharogenic Amylase Fraction—Diagram 1 summarizes the fractionation procedure of the saccharogenic amylase fraction.

As shown in Table IV, the preparation (Fraction I) obtained from the precipitate between 0.25 and 0.8 saturation with $(NH_4)_2SO_4$ possesses the properties of being precipitated with rivanol and adsorbed on corn starch at pH 3.6. On the other hand, Fraction II obtained from the supernatant of 0.8 saturation with $(NH_4)_2SO_4$ possesses the reverse properties of being not precipitated

DIAGRAM I

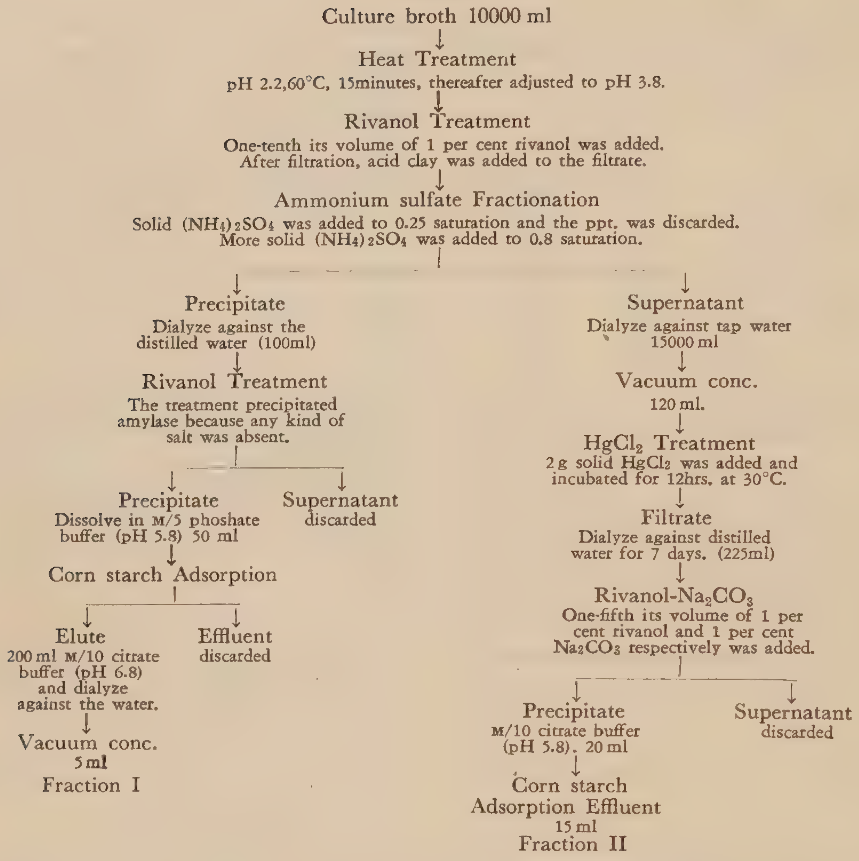

Culture broth 10000 ml
Heat Treatment
pH 2.2,60°C, 15minutes, thereafter adjusted to pH 3.8.
Rivanol Treatment
One-tenth its volume of 1 per cent rivanol was added. After filtration, acid clay was added to the filtrate.
Ammonium sulfate Fractionation
Solid $(NH_4)_2SO_4$ was added to 0.25 saturation and the ppt. was discarded. More solid $(NH_4)_2SO_4$ was added to 0.8 saturation.
Precipitate
Dialyze against the distilled water (100ml)
Rivanol Treatment
The treatment precipitated amylase because any kind of salt was absent.
Precipitate
Dissolve in M/5 phoshate buffer (pH 5.8) 50 ml
Supernatant
discarded
Corn starch Adsorption
Elute
200 ml M/10 citrate buffer (pH 6.8) and dialyze against the water.
Effluent
discarded
Vacuum conc.
5 ml
Fraction I
Supernatant
Dialyze against tap water 15000 ml
Vacuum conc.
120 ml.
$HgCl_2$ Treatment
2 g solid $HgCl_2$ was added and incubated for 12hrs. at 30°C.
Filtrate
Dialyze against distilled water for 7 days. (225ml)
Rivanol-Na_2CO_3
One-fifth its volume of 1 per cent rivanol and 1 per cent Na_2CO_3 respectively was added.
Precipitate
M/10 citrate buffer (pH 5.8). 20 ml
Supernatant
discarded
Corn starch Adsorption Effluent
15 ml
Fraction II

TABLE IV

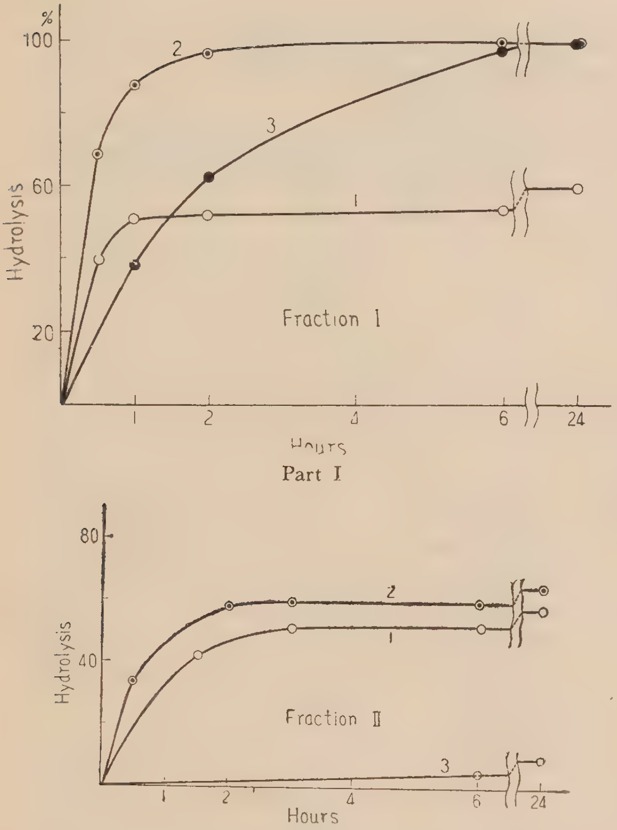
PURIFICATION OF SACCHAROGENIC AMYLASE FRACTION. FRACTION I.

Precipitate in 0.8 saturation with $(NH_4)_2SO_4$

Treatment	Volume ml	U.N./ml	U.N./N mg	Yield
Original Enzyme Solution	10000	3.5	54	100
Heat Treatment	10400	0.3	78	9
Rivanol Treatment	9800	0.25	–	7.5
Ammonium sulfate (ppt. in 0.8 saturation)	100	20	1000	5.8
Rivanol Precipitate	50	30	2300	4.3
Corn Starch Adsorption	5	120	2500	1.8

FRACTION II. Supernatant in 0.8 saturation with $(NH_4)_2SO_4$

Treatment	Volume ml	U.N./ml	U.N./N mg	Yield
Supernatant in 0.8 satu.	1300	–	–	
Vacuum concentration	120	6	52	100
$HgCl_2$ Treatment	135	4.5	80	84
Rivanol-Na_2CO_3 Treatment	20	18	110	50
Corn Starch Treatment	15	16	110	33



Part I

Part II

FIG. 2. Comparison in Hydrolysis of Amylaceous Materials by Fraction I (Part I) with that by Fraction II (Part II).

Hydrolysis curve in both figures: Curve 1; on soluble starch. Curve 2; on glutinous rice starch. Curve 3; on β-LD (glycogen). The reaction mixture contained 10.0 mg of substrate, 2.6 units of saccharogenic amylase fraction I (Part I) or 1.6 units of saccharogenic amylase fraction II (Part II); volume 20 ml.

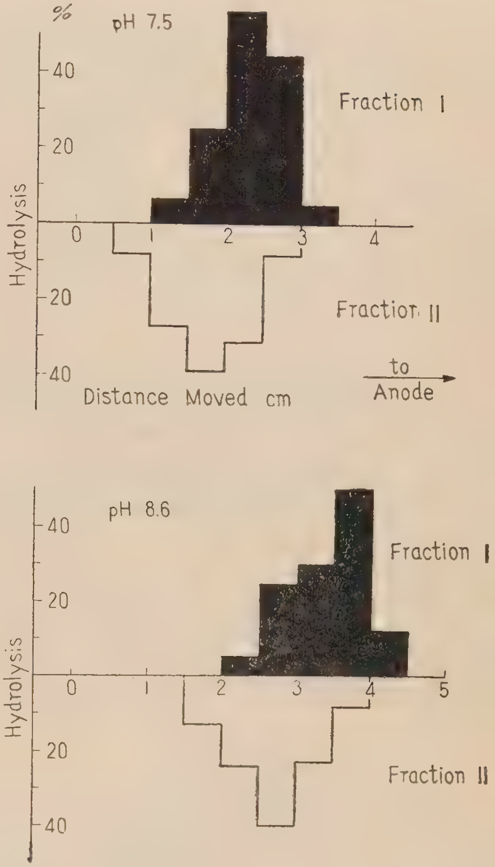


FIG. 3. Mobility comparison of Fraction I with II in paper electrophoresis.

Hydrolysis per cent after 48 hrs. at 30°C was shown as amylase activity. For the procedure, see the text.

with rivanol alone, and not adsorbed on corn starch. But it is precipitated with rivanol and Na_2CO_3. The enzymatic properties of these two preparations were investigated with soluble starch, glutinous rice starch and β-LD from glycogen. As illustrated in Fig. 2, Fraction I hydrolyzes glutinous rice starch and β-LD from glycogen completely to glucose, but in case of soluble starch incompletely. Fraction II hydrolyzes glutinous rice starch into glucose up to an extent of 60–65 per cent, soluble starch up to 60 per cent and β-LD from glycogen up to only 10 per cent of theory. It has been proved from this experimental result that Fraction II has no debranching activity.

Furthermore, two preparations were examined by filter paper electrophoresis. In this case, the strip was cut down into pieces of 0.5-cm in width. As it is obvious from Fig. 3, Fraction I moved faster to anode than Fraction II. Comparing the β-LD hydrolyzing activity with that of the saccharogenic amylase on each of the 9 pieces cut out from the paper electrophoretic diagram of Fraction I (pH 8.6), the ratio of both ac-

TABLE V

THE SACCHAROGENIC AND β-LD HYDROLYZING ACTIVITIES OF EACH 9 PIECES CUT OUT OF THE STRIP OF FRACTION I

	No.**	4		5		6		7		8		9	
	Substrate / Hours	Soluble starch	β-LD	Soluble starch	β-LD	Soluble starch	β-LD	Soluble starch	β-LD	Soluble starch	β-LD	Soluble starch	β-LD
Hydrolysis %	48	0	0	4.5	–	25.2	11.6	30.2	14.4	49.6	38.0	1.20	6.1
	72	0	0	6.8	3.0	38.0	17.7	41.4	19.6	52.2	61.3	18.2	8.8
Ratio	48	–		–		1 : 0.46		1 : 0.47		1 : 0.76*		1 : 0.50	
	72	–		1 : 0.44		1 : 0.46		1 : 0.47		1 : 1.17*		1 : 0.48	

** Number shows that of pieces cut out in 0.5cm wide from the starting line toward anode.

* These figures should be excluded from the comparison because their corresponding hydrolysis per cent on soluble starch was approaching to the limiting extent.

tivities was found to be almost constant (1: 0.44 to 0.50) over the whole-range of enzyme active pieces (Table V).

From these results, it is reasonable to infer that the saccharogenic amylase fraction consists of a saccharogenic amylase with debranching activity and another saccharogenic amylase which is deficient in debranching activity.

DISCUSSION

The data in this communication emphasize that two different saccharogenic amylases, I and II may exist in the saccharogenic amylase fraction.

Saccharogenic amylase I, hydrolyzes β-LD from glycogen and glutinous rice starch completely, whereas saccharogenic amylase II, in turn, has essentially no activity against β-LD. As reported previously[8], saccharogenic amylase I which is distinguished in adsorbability on raw corn starch plays a significant role in its complementary action toward the dextrinogenic amylase fraction on raw starch digestion, whilst saccharogenic amylase II has no trace of such action.

Furthermore, as described above, the properties of saccharogenic amylase I in regard to adsorbability on raw starch, precipitability with rivanol, and the attitude against paper electrophoresis are quite different from that of amylase II.

From these results, it is plausible to consider that the saccharogenic amylase fraction may consist of these two different amylases rather than saccharogenic amylase plus some other debranching factor such as amylo-1,6-glucosidase or oligo-1,6-glucosidase.

By using a certain technique of paper electrophoresis, Ando[6] has presented evidence that two different saccharogenic amylases exist in the mold bran extract of *Aspergillus usamii*, but further investigation of this problem has not yet been performed.

Fukumoto et al.[9], obtained the crystalline gluc amylase from *Rhizopus delemar*, which shows a close resemblance to the saccharogenic amylase I prepared by the author, except that the extent of hydrolysis in soluble starch by the former, is rather higher than that by the latter.

From Taka-diastase, Okazaki[10] prepared a highly-purified glucose-producing amylase, tentatively referred to as "Taka-diastase B", which is reported to remain in the supernatant in the $(NH_4)_2SO_4$ saturation and also in the rivanol treatment. These properties are similar to those of saccharogenic amylase II, prepared by the auther.

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[8] S. Ueda, This Bulletin, **21**, 284 (1957).

[9] J. Fukumoto, Y. Tsuzisaka and K. Minamii, *Symposia on Enzyme Chem. (Japan)*, **9**, 94 (1954).

[10] H. Okazaki, *J. Agr. Chem. Soc. Japan*, **28**, 48, 51 (1954), **29**, 181 (1955).

As to the question whether saccharogenic amylase I is either only one enzyme possessing a dual-activity or a mixture of coexisting different enzymes, it is very difficult to decide, because heretofore many attempts to separate saccharogenic activity from debranching activity have resulted in fail.

SUMMARY

The following results were obtained from the fractionation of the saccharogenic amylase fraction:

(1) The debranching active principle precipitates at a lower concentration of ammonium sulfate than in the case of saccharogenic amylase.

(2) In paper electrophoresis, the debranching active principle readily moves to anode.

(3) In fractionation with $(NH_4)_2SO_4$ as well as in paper electrophoresis, debranching activity, free from saccharogenic activity could not be observed.

(4) From the results of electrophoresis, rivanol treatment and corn starch adsorption, it has been shown that the saccharogenic amylase fraction contains two types of saccharogenic amylases one possessing debranching activity and the other not.

Acknowledgement. The author expresses his sincere thanks to Professor I. Yamasaki for his encouragement and interest in this work. This study was aided in part by a grant from the Scientific Research Fund, Ministry of Education.

Crystallization of Black-Koji Amylases

Sir:

Ueda[1] has prepared saccharogenic and detrinogenic amylase fractions from Black-Koji amylase system and conducted studies on the specific amylolytic actions of each preparation.

The author has succeeded in the crystallization of both saccharogenic and dextrinogenic amylases from the culture solution of *Asp. awamori* var. Kawachii strain of black Aspergillus. The former crystalline amylase hydrolyzed potato starch to an extent of 90% of theory, and produced glucose and dextrin which were stained purple with iodine, but the latter crystalline amylase was hydrolyzed to an extent of 62%, and produced glucose, maltose and achromatic dextrin.

The mold was cultured for 3 days at 30°C in a 100-liter-capacity fermenter, with 70 l-aeration per minute and 180 r.p.m. agitation, in 80 l of the medium containing 3% potato starch, 1% CH_3COONH_4, 0.3% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.05% NaCl.

Saccharogenic amylase was purified in a crystalline from in the following manner. Amylase produced in 200 l of the culture filtrate was concentrated in 2 l by repeating twice ammonium sulfate precipitation at the concentration range of from 30 to 60%, pH 5. The concentrated enzyme solution was treated at 0°C with acid, followed by alkali; these treatments preferentially destroyed the dextrinogenic amylase. The concentrate was brought to pH 1.8 by the addition of 2 M H_2SO_4, held at 0°C for 3 day, and the precipitated material was filtered off in the ice-room; it was then adjusted to pH 10.0 by the addition of 2 M NH_4OH, held at 0°C overnight, and neutralized to pH 5 with 2 M H_2SO_4. Then, the solid ammonium sulfate was added up to a concentration of 30%, and the produced precipitate discarded. The supernatant was treated with a further amount of solid ammonium sulfate up to a concentration of 50%, and the precipitate filtered using Hyflo Super Cel. The precipitate was dissolved in 400 ml distilled water, and at first, dialyzed against running water for 2 days, then against distilled water for 1 day. At the time dialysis was completed, no sulfate could be found in the outer-liquid. An amount of 55 ml of 2% rivanol solution was added to the dialyzed supernatant, and the produced precipitate was collected on the centrifuge and it was dissolved in M/2 acetate buffer at pH 6, making it free from a small amount of black insoluble material by centrifugation and filtration. After removing rivanol with acid clay, the enzyme solution was dialyzed against distilled water for 2 days in the ice-room. Subsequently, the rivanol treatment was repeated, and the resulting precipitate was dissolved in M/2 acetate buffer at pH 5. The clear solution was then fractionally precipitated with ammonium sulfate at concentrations of from 35 to 45%, and the precipitate dissolved in distilled water.

The ammonium sulfate fractionation was repeated in the same fashion, and the 37 to 43% fraction was dissolved in a minimum quantity of 0.01 M sodium acetate solution. A small quantity of ammonium sulfate was added to the outer-dialyzate every day, until crystallization occurred, requiring about 10 days. The crystalline amylase was recrystallized in the same way mentioned above.

Dextrinogenic amylase was purified in the crystalline form described in the following procedure. The enzyme formed in 40 l of the culture filtrate was concentrated in 400 ml by ammonium sulfate precipitation in the same way as the saccharogenic amylase. The concentrate was dialyzed against running

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[1] S. Ueda, This Bulletin, **20**, 148 (1956).

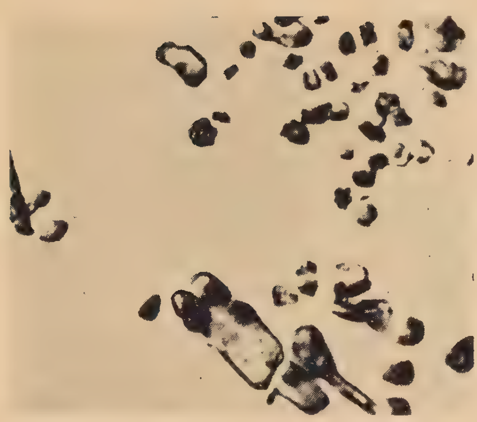

FIG. 1. Crystalline Saccharogenic Amylase from Black-Koji Mold.

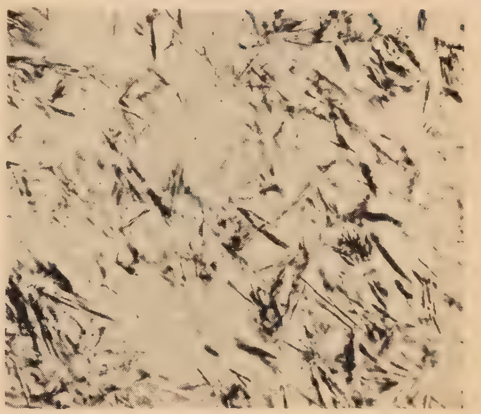

FIG. 2. Crystalline Dextrinogenic Amylase from Black-Koji Mold.

water for 2 days. To 900 ml of the dialyzed solution, 45 ml of 2 M $Ca(CH_3COO)_2$ was added, and the precipitate was filtered off. Acetone was added to the filtrate up to a concentration of 30%, and held at 0°C overnight. After discarding the precipitate, acetone was added further to the supernatant up to a concentration of 65%, and the mixture was held at 0°C overnight. The precipitate was dissolved in 400 ml distilled water, then centrifuged and discarded a mixture of the insoluble material, and 250 g of ammonium sulfate was added to the supernatant. The precipitate was dissolved in 100 ml distilled water, dialyzed against running water for 2 days and then against distilled water for 1 day successively.

An quantity of 13 ml of 2% rivanol solution was added to the dialyzate and the precipitate thus produced was dissolved in 50 ml of M/2 acetate buffer at pH 5, and the insoluble material was discarded. After removal of the rivanol with acid clay, adding acetone to the supernatant to a concentration of 30%, the mixture was held at 0°C for 3 days. The precipitate (mostly consisting of the denatured saccharogenic amylase) was discarded, and acetone was further added, until a 55% concentration was attained. The precipitate (mostly consisting of the active dextrinogenic amylase) was dissolved in 20 ml of 0.02 M calcium acetate, and after acetone fractionation was repeated twice, the precipitate formed between 35 and 54% acetone was collected, dissolved in a minimum quantity of 0.02 M calcium acetate, and then acetone was added to it until a faint turbidity appeared (about 32%), and the mixture set away at 0°C. The enzyme obtained crystallized within 2 to 3 days.

No activity of phosphatase, α-amylase and protease was recognized in the crystalline saccharogenic amylase. Also no activity of maltase and protease was observed in the crystalline dextrinogenic amylase. From their pattern of electrophoresis, both amylases seemed to have one component respectively, and their isoelectric points were found to be around 3.6. The optimum pH of the saccharogenic amylase was 4.0, and that of dextrinogenic amylase 5.2.

The author wishes to thank Prof. I. Yamasaki of Kyushu University, for his constant guidance throughout this work. The author also wishes to thank the Nihon-Shurui Co. for permission to prepare the crude enzyme solution in the Research Laboratory of that Co., Kumamoto.

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Received September 2, 1957

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 388~389, 1957]

Structure of Sawamilletin from Sawamillet Oil

Sir:

The deposition of a colorless and hexagonal crystal in an acetone solution of sawamillet (*Echinochloa crus gallis* L.) oil was found by us and was named Sawamilletin ($C_{31}H_{52}O$ m.p. 278°$[\alpha]_D+8.2°$). Sawamilletin has a methoxy radical. Ultraviolet and infrared absorption spectra of this compound were investigated, but these did not indicate the presence of the carbonyl radical and conjugate bond in their structure. However, they had absorptions at 6.15 μ and 12.25 μ; therefore, we believe that sawamilletin has a trisubstituted double bond of the type: $>C:C<_{H}$ [1].

The perbenzoic acid oxidation in chloroform solution of sawamilletin yielded the oxide. Sawamilletin oxide (III) [$C_{31}H_{52}O_2$ m.p. 218.5–219.5°$[\alpha]_D+47°$] has neither color reaction with tetranitromethane nor absorption at 12.25 μ in its infrared spectrum. We therefore concluded that sawamilletin was a pentacyclic compound having a trisubstituted double bond and a methoxy radical.

Isomerization of sawamilletin with dry hydrogen chloride in chloroform afforded isosawamilletin (II) [$C_{31}H_{52}O$ m.p. 247°–8°$[\alpha]_D+92.4°$], which was identical with the methyl ether of β-amyrin.[2]

Because sawamilletin and isosawamilletin exhibited a point of inflexion at 9.1 μ[3] in the infrared region and also by taking into consideration the Morice-Simpson synthesis of methyl ether of β-amyrin[2], the methoxy radical is equatorial (β). Thus, isosawamilletin was designated as 3β-methoxyolean-12-ene.

The acid-catalysed rearrangement of sawamilletin oxide (III) gave an isomerized compound (IV) [$C_{31}H_{52}O_2$ m.p. 230°–231.5° $[\alpha]_D+79.4°$], which had color reaction with tetranitromethane and had a characteristic absorption band at 12.25 μ in the I.R. region, and an acetate (V) [$C_{33}H_{54}O_3$ m.p. 169°–170.5° $[\alpha]_D+62.8°$], was obtained by treatment of the aceticanhydride.

It was confirmed that the isomerized substance of sawamilletin oxide was an unsaturated alcohol (IV).

The chromium trioxide oxidation of this unsaturated alcohol (IV) afforded a ketone (VI) [$C_{31}H_{50}O_2$ m.p. 245°–7°$[\alpha]_D+26.2°$] which indicated the presence of the trisubstituted double bond and nonconjugated carbonyl bond in their infrared and ultraviolet absorption spectrum.

It was deduced from the foregoing experimental results that the double bond and the hydroxy radical in the unsaturated alcohol (IV) were not present in the same ring.

In consideration of the properties of many other triterpenoids[4,5], the double bond of sawamilletin derivatives except sawamilletin itself, is located at position 12:13, thence we presume that the sawamilletin derivatives have the same structures with the amyrin in the stereochemical sense.

On the other hand, the molecular rotation differences prove that the carbonyl radical is located on either B- or D-ring, not on A-, C- and E-ring[5,6]. We concluded that configuration and conformation of the hydroxy group in the unsaturated alcohol (IV) was at 15-α by comparison with optical rotation of Skimmiol (Taraxerol) derivatives[5] and sawamilletin derivatives.

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[1] Ruzicka, O. Jeger et al.; *Helv., Chim. Acta*, **33** 672, 687, 711, 1050 (1950).

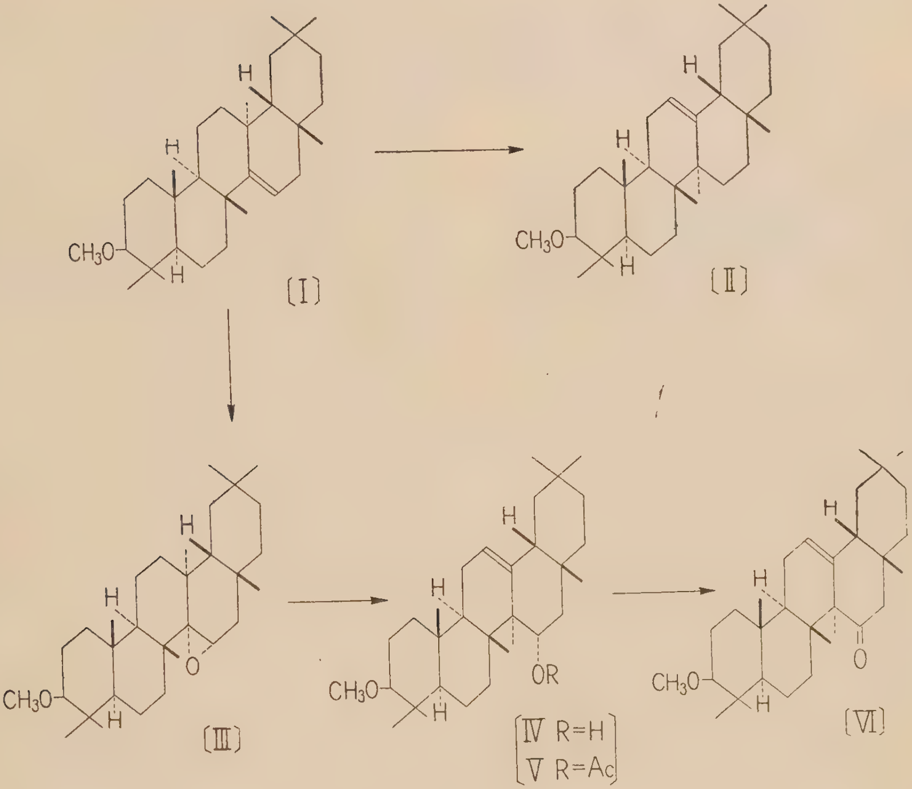
[2] Isabel M. Morice and James C.E. Simpson; *J. Chem. Soc.*, **1942**, 198.

[3] J.E. Page; *J. Chem. Soc.*, **1955**, 2017.

[4] D.H.R. Barton and C.J.W. Brooks; *J. Chem. Soc.*, **1951**, 257. E.J. Corey and J.J. Ursprung; *J. Am. Chem. Soc.*, **78**, 5041 (1956). George Brownlie, M.B.F. Fayez, F.S. Spring, Robert Stevenson and W.S. Struchan; *J. Chem. Soc.*, **1956**, 1377.

[5] J.M. Beaton, F.S. Spring, Robert Stevenson and J.L. Stewart: *J. Chem. Soc.*, **1955**, 2131.

[6] W. Klyne; *J. Chem. Soc.*, **1952** 2916, **1954**, 1979. William M. Stokes, Werner Bergmann; *J. Org. Chem.*, **17**, 1194 (1952). Ruzicka, Crob, Egli and O. Jeger; *Helv. Chim. Acta*, **26**, 1218 (1943). B. Bischof and O. Jeger; *Helv. Chim. Acta*, **31**, 1205 (1948).



Consequently, it was found that sawamilletin was 3β-methoxy-isoolean-14-ene, oxide (III) was 3β-methoxy-14α-15α-epoxy-isooleane, unsaturated alcohol (IV) was 3β-methoxy-olean-12-en-15α-ol and ketone (VI) was 3β-methoxy-olean-12-en-15-one as indicated in the above Figure.

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Hydroxylation of Steroids at C-11 Position by Fungi belonging to the Genus Corticium

Sir:

We wish to report on a few micro-organisms which are able to hydroxylate steroids at C-11 position. For the last several years, we have conducted studies on the ability of steroid oxidations of the fungi belonging to Basidiomycetes and found several strains in genus Corticium to be able to transform 17α-hydroxydesoxycorticosterone to hydrocortisone and epi-hydrocortisone*. These were *Corticium sasakii*, *C. microsclerotia*, *C. praticola*, etc. and among them, especially, *C. sasakii* demonstrated an excellent yield of hydrocortisone. Exner1) included them, except *C. praticola*, in a species Pellicularia filamentosa, but some of the other strains of this species could scarcely hydroxylate at C-11β position. This is an interesting fact from the taxonomical point of view. Recently, Greenspan et al.2), touched lightly upon a fact that some species of Rhizoctonia could hydroxylate steroids at C-11 position, although its details were not montioned in their communication.

We present one of our experimental results obtained with a strain of *C. sasakii*, IFO-No. 5254. Fifty ml of a sterilized medium ($NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1 g, Potato, 50 g; Wheat starch, 30 g; Tap water, 1000 ml.) in each 250 ml shaking flask was inoculated with 5 ml of vegetative mycelium, which was incubated on a reciprocating shaker for 5 days at 28°C. At the end of this period, 17α-hydroxydesoxycorticosterone was added as an ethanolic solution, and the incubation was further continued for 48 hours. The transformation products were extracted from the broth by ethyl acetate and the extract was concentrated to dryness under reduced pressure in a stream of nitrogen. The steroids present in the extract were separated by paper partition chromatography and detected as several spots by means of their absorption of ultraviolet ray at 240 mμ.

For the purpose of their identification, the transformation products were separated by adsorption chromatography, and thus the compounds, I, II and III were gained. I was recrystallized from acetone; m.p. 205–207°C, $[\alpha]^{25}_{D}+162°$ (ethyl alcohol). Its elemental analysis afforded a formula, $C_{21}H_{30}O_5$. Monoacetate of I; m.p. 214–217°C, $[\alpha]^{27}_{D}+162°$ (chloroform). In addition to these constants their infrared spectra and chromatographic behaviors were identical with those of the authentic samples of hydrocortisone and its monoacetate. II was recrystallized from chloroform followed by methanol; m.p. 212–216°C. $[\alpha]^{20}_{D}+120°$ (ethyl alcohol). Its elemental analysis afforded a formula, $C_{21}H_{30}O_5$, Diacetate of II; m.p. 201–203°C, $[\alpha]_{D}+120°$ (chloroform). Furthermore, comparison of the infrared spectra and chromatographic behaviors of II and its derivative with those of the authentic samples of epi-hydrocortisone confirmed the identity. III was recrystallized from methanol, m.p. 233–236°C. $[\alpha]^{18}_{D}+144°$ (ethyl alcohol), $\lambda_{max.}$ 243.5 mμ (ε 15500). Its elemental analysis gave it the formula, $C_{21}H_{30}O_5$. Diacetate of III; m.p. 190–191°C. The above results showed that compound III was a monohydroxyderivative of 17α-desoxycorticosterone and the structure of it is now under investigation.

Two other transformation products were found on the paperchromatogram in small quantities, and are now under investigation. Besides 17α-hydroxydesoxycorticosterone, desoxycorticosterone, progesterone and 1-dehydro-17α-hydroxydesoxycorticosterone were also able to be hydroxylated at C-11 position by this organism. Complete details will be presented in this Bulletin and the Journal of the Pharmaceutical Society of Japan.

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* These results were filed as a patent application in November, 1956.

1) B. Exner; *Mycologia*, **45**, 698 (1953).

2) G. Greenspan et al.; *J. Am. Chem. Soc.*, **79**, 3922 (1957).

The authors wish to express their sincerest thanks to Dr. S. Kuwada, Dr. K. Sato and Dr. T. Matsukawa of our institutes for their guidance and encouragement throughout this work, and also to Dr. B. Exner, Louisiana State University, and Dr. A. H. S. Brown, Commonwealth Mycological Institute, for kindly sending us the strains.

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On the Xylanase System of *Aspergillus batatae*

Sir:

By investigating the process of saccharification of xylan caused by various species of bacteria and molds we have found that there are several types of xylanase systems in these organisms[1]. In general, bacterial xylanase system consists merely of liquefying enzymes[2], while that of molds is composed of a varied proportion of liquefying and saccharifying enzymes, which may be designated by the name xylanase A and xylanase B, respectively, hereafter.

These two enzymes were isolated from the culture of *Aspergillus batatae* which has been found to show the most powerful xylanase activity among more than one hundred strains of microorganisms tested. From the wheat-bran culture of the mold, xylanase A was obtained in crystalline form in a yield of 15 mg per 1.5 kg of the culture. Xylanase B was also obtained in a fairly purified state, and electrophoresis revealed this preparation to be free from xylanase A and composed of three components.

Xylanase A has a property of hydrolysing β-1,4-xylosidic linkages of polyxylosaccharides at random. When xylan is used as the substrate, the latter is subjected to a rapid decrease of molecular weight and viscosity with only an insignificant increase of the reducing power. By comparing the action of this enzyme upon various oligosaccharides of different grades of polymerization, it was found that the higher the degree of polymerization, the faster proceeded the hydrolysis. The final products resulted from xylan were identified[3] to be xylotriose, xyloarabinoside, xylobiose, limit polyxylosan and small amounts of xylotetraose and L-arabinose, while no D-xylose was detectable. From xylohexaose also the following substances—xylotetraose, xylotriose and xylobiose—were produced.

Xylanase B attacks poly- and oligoxylosaccharides from the end of β-1,4-xylosidic linkages and liberates D-xylose. When xylan is used as the substrate, there is only a slight decrease in viscosity and the reducing power increase remarkably with the liberation of D-xylose which emerges even in the initial stage of the reaction. From xylohexaose likewise, large amounts of D-xylose and xylopentaose, xylotetraose and other oligosaccharides were produced. On acting upon xylobiose or xylotriose, this preparation produces xylosaccharides having higher molecu-

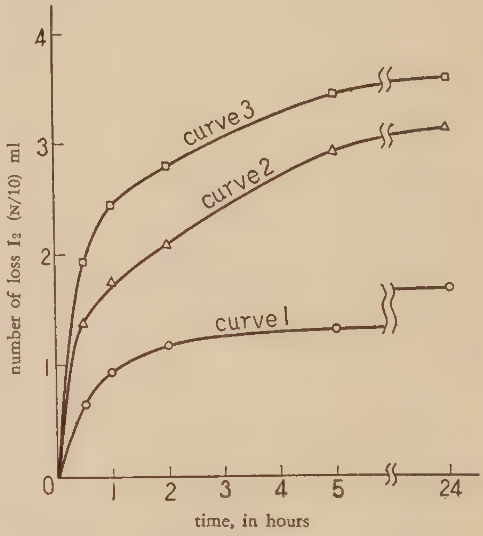

FIG. 1. Saccharifying Curves on Xylan

curve 1—xylanase A
curve 2—xylanase B
curve 3—the joint system

Reaction media: 2% xylan solution 12.5 ml; M/10 acetate buffer, pH 5.0, 5 ml; enzyme solution + dist. water 7.5 ml.

Reducing power: Number of loss I_2-solution (N/10) ml per reaction medium 5 ml.

Reaction temp. 50°C.

Enzymes: Sufficient amounts were used. Xylanase A, 1.5 saccharifying units, and xylanase B 2.3 saccharifying units.

---

[1] Sakuzo Fukui; Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, March 30, 1955.

[2] Sakuzo Fukui and Masao Yoshioka; Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, April 1, 1956.

[3] Sakuzo Fukui; unpublished work.

lar weights than the substrates, a fact suggesting that the preparation has an activity of *trans*-xylosidase.

These findings described above show that the modes of xylanases A and B on xylan are very similar to those of α- and β-amylase, respectively, in their action upon starch. The joint action of xylanases A and B may be of importance in the process of saccharification of xylan, and as a matter of fact, the experimental results presented in Fig. 1 show that in the presence of both xylanases A and B the saccharification of xylan occurred more profoundly than it was the case when either one of the enzymes was present. The experiments now in progress in our laboratory have provided the evidence that *Aspergillus batatae* containing these two enzymes can successfully be used as a saccharifyer for lactic acid fermentation using xylan as the material[4].

We wish to express our thanks to Prof. K. Kitahara for his guidance throughout this work, and to Dr. R.L. Whistler for his kindness of supplying us the preparation of oligoxylosaccharides.

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Received September 30, 1957

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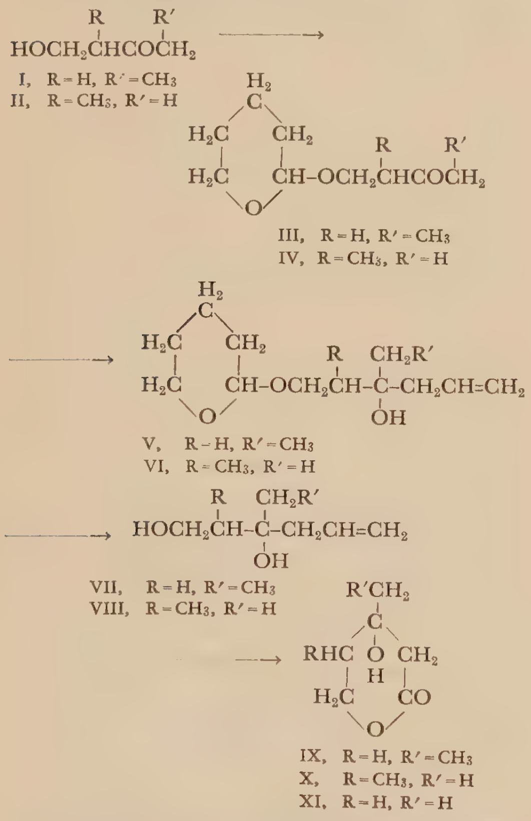
[4] Sakuzo Fukui, Akira Ôi and Akira Ôbayashi; Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, April 11, 1957.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 6, p. 394~395, 1957]

Synthesis of Some Homologs of D,L-β-Hydroxy-β-methyl-δ-valerolactone

Sir:

β-Hydroxy-β-methyl-δ-valerolactone (XI) has been shown to be a new acetate-replacing factor for lactobacilli[1]. At about the same time, G. Tamura has recently isolated a new growth factor for *Lactobacillus homohiochi* and *Lactobacillus heterohiochi* from the culture-broth of *Aspergillus oryzae*[2] and confirmed its identity with the above lactone[3]. In order to find a new antimetabolite for lactobacilli we have synthesized some homologs of XI via the following procedures.



I, R=H, R′=CH3
II, R=CH3, R′=H

III, R=H, R′=CH3
IV, R=CH3, R′=H

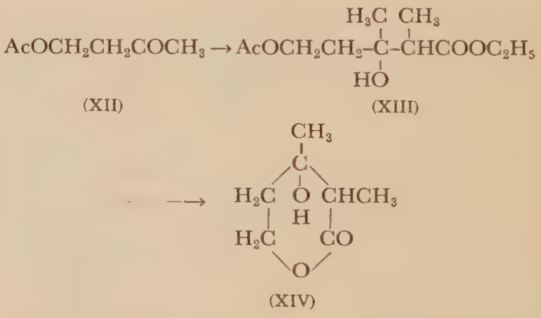
V, R=H, R′=CH3
VI, R=CH3, R′=H

VII, R=H, R′=CH3
VIII, R=CH3, R′=H

IX, R=H, R′=CH3
X, R=CH3, R′=H
XI, R=H, R′=H

β-Ethyl-β-hydroxy-δ-valerolactone (IX) and β,γ-dimethyl-β-hydroxy-δ-valerolactone (X) were prepared respectively by the similar route as that for XI described in the previous paper[4]. The boiling points and analytical data[5] of the compounds (I–X) are given below in Table I.

α,β-Dimethyl-β-hydroxy-δ-valerolactone (XIV) was synthesized by the following method, almost similar to that of Hoffman et al. for XI[6]. The Reformatsky reaction between 4-acetoxy-2-butanone (XII) and ethyl α-bromopropionate gave ethyl 5-acetoxy-2,3-dimethyl-3-hydroxypentanoate (XIII), b.p. 125–129°/2 mm. *Anal.* Calcd. for $C_{11}H_{20}O_5$: C, 56.88; H, 8.68. Found: C, 57.00; H, 8.55. XIII was hydrolyzed with sodium hydroxide in ethanol-water and acidified. XIV was then taken up in chloroform and distilled at 140°/2 mm. *Anal.* Calcd. for $C_7H_{12}O_3$: C, 58.31; H, 8.39. Found: C, 58.34; H, 8.09.



(XII)
(XIII)
(XIV)

By the preliminary test it has been found that α,β-dimethyl-β-hydroxy-δ-valerolactone inhibits the growth of *Lactobacillus heterohiochi* in the presence of β-hydroxy-β-methyl-δ-

---

1) D. Wolf, C. Hoffman, P. Aldrich, H. Skeggs, L. Wright and K. Folkers, *J. Am. Chem. Soc.*, **78**, 4499 (1956).

2) G. Tamura, *J. Gener. Appl. Micob.*, **2**, 431 (1956).

3) G. Tamura, This Bulletin, **21**,202 (1957).

4) S. Tamura and M. Takai, *ibid.*, **21**, 260 (1957).

5) The microanalyses reported herein were carried out by members of the Analytical Laboratory of this Department, to whom we are grateful.

6) C. Hoffman, A. Wagner, A. Wilson, E. Walton, C. Shunk, D. Wolf, F. Holly and K. Folkers, *J. Am. Chem. Soc.*, **79**, 2316 (1957).

TABLE I
BOILING POINTS AND ANALYTICAL DATA OF THE COMPOUNDS

Compd.	b.p., C°.	Formula	Carbon, %		Hydrogen, %	
			Calcd.	Found	Calcd.	Found
I	71– 72 (7 mm.)	$C_5H_{10}O_2$	58.80	58.62	9.87	9.95
II	88– 89 (20 mm.)	$C_5H_{10}O_2$	58.80	58.71	9.87	9.98
III	93– 95 (3 mm.)	$C_{10}H_{18}O_3$	64.49	64.35	9.74	9.85
IV	96– 97 (5 mm.)	$C_{10}H_{18}O_3$	64.49	64.56	9.74	9.92
V	111–113 (5 mm.)	$C_{13}H_{24}O_3$	68.38	68.10	10.59	10.82
VI	124–127 (5 mm.)	$C_{13}H_{24}O_3$	68.38	68.40	10.59	10.74
VII	104–106 (4 mm.)	$C_8H_{16}O_2$	66.63	66.83	11.18	10.96
VIII	115–117 (7 mm.)	$C_8H_{16}O_2$	66.63	66.67	11.18	11.43
IX	130–134 (2 mm.)	$C_7H_{12}O_2$	58.31	58.34	8.39	8.63
X	133–137 (2 mm.)	$C_7H_{12}O_2$	58.31	58.19	8.39	8.70

valerolactone[7]. Detail studies will be reported later.

We wish to express our sincerest thanks to Prof. Y. Sumiki for his guidance and encouragement throughout the course of these experiments.

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[7] Indebts are due to Mr. G. Tamura for the biological test.

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A New Gibberellin, Gibberellin A_4

Biochemical Studies on "Bakanae" Fungus. Part XLII

Sirs:

In this communication, we publish the isolation and characterization of a new gibberellin which is named gibberellin A_4.

When the crude crystals of methylester of gibberellins obtained from the broth filtrate of tank culture incubating the strain No. 4 or G-4 of *Gibberella fugikuroi* in the medium composed of glycerol, ammonium chloride, monopotassium phosphate, tartaric acid, magnesium sulphate, zinc sulphate and ferric sulphate were subjected to the chromatography of alumina using a mixture of benzene-ethylacetate (5:1 by vol.) as the solvent system, a new methylester, m.p. 175–6°, different from methylesters of gibberellins A_1, A_2 and gibberellic acid, namely A_3, was obtained from the earlier eluted fraction than all known gibberellins fractions in relatively low yield. The partition chromatography through silicic acid was attempted for the purpose of obtaining the new free acid, using one mole phosphate buffer of pH 5.4 as the stationary phase and chloroform-ethylacetate mixture (1:1 by vol.) as the mobile phase and we isolated the free acid, d.p. 222°, from, also, the earlier eluted fraction. The methylester of this free acid is identical in all respects with the methylester obtained from the chromatography of alumina described above. This new acid shows the characteristic physiological activity in about the same degree as gibberellin A_1 by our testing method against rice seedlings but inhibits the growth of root evidently.

We named this substance gibberellin A_4 and $C_{18}H_{22}O_5$ or $C_{19}H_{24}O_5$ was assigned as its molecular formula from the analytical data of itself and its derivatives and molecular weight determination. The further studies are now undergoing to determine which of above possible formulae is correct. Gibberellin A_4, $C_{18}H_{22}O_5$ or $C_{19}H_{24}O_5$, melts at 222° with decomposition and has an optical rotation $[\alpha]_D^{20} -20.8°$ (c=0.34, CH_3OH); Calcd. for $C_{18}H_{22}O_5$, C 67.91%, H 6.97%, M.W. 318; for $C_{19}H_{24}O_5$, C 68.65%, H 7.28%, M.W. 332; Found, C 67.55%, H 7.35%, M.W. 334 (titration). Gibberellin A_4 monomethylester, $C_{19}H_{24}O_5$ or $C_{20}H_{26}O_5$, melts at 175–6°; Calcd. for $C_{19}H_{24}O_5$, C 68.65%, H 7.28%, OCH_3 8.90%, M.W. 332; for $C_{20}H_{26}O_5$, C 69.34%, H 7.57%,

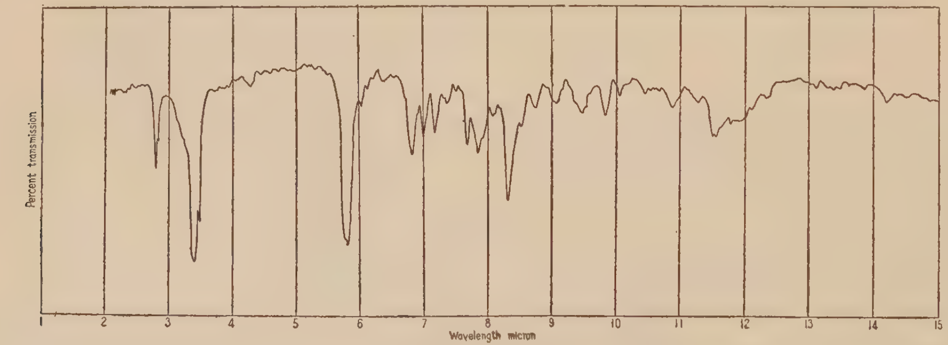

FIG. 1.
Gibberellin A_4

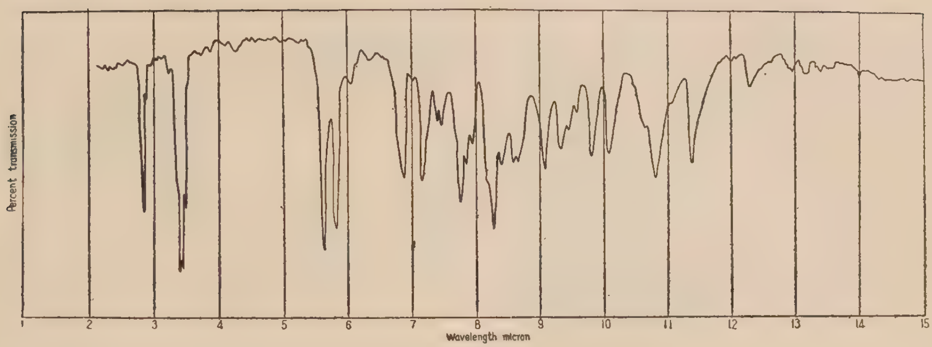

FIG. 2.
Gibberellin A_4 monomethylester

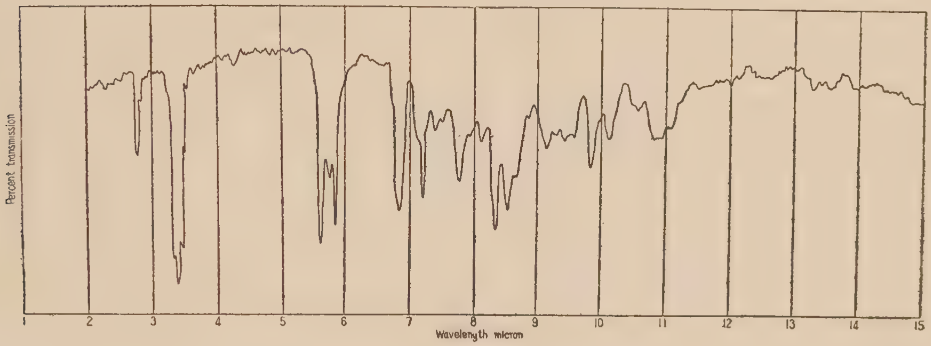

FIG. 3.
Dihydrogibberellin A_4 monomethylester

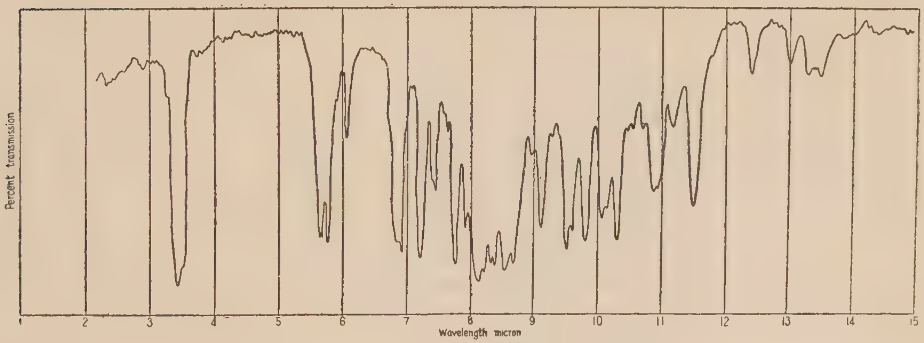

FIG. 4.
Monoacetyl gibberellin A_4 monomethylester

OCH_3 8.52%, M.W. 346; Found, C 69.01%, H 7.28%, OCH_3 8.09%, M.W. 342 (by Rast).

Gibberellin A_4 monomethylester gives a dihydrogibberellin A_4 methylester, m.p. 141°, $C_{19}H_{26}O_5$ or $C_{20}H_{28}O_5$ over Adams' catalyst (Calcd. for $C_{19}H_{26}O_5$, C 68.24%, H 7.84%; for $C_{20}H_{28}O_5$, C 68.94%, H 8.10%; Found, C 68.63%, H 8.02%) and a monoacetyl gibberellin A_4

monomethylester, $C_{21}H_{26}O_6$ or $C_{22}H_{28}O_6$, m.p. 131–2°, by treatment of pyridine- acetic anhydride at room temperature (Calcd. for $C_{21}H_{26}O_6$, C 67.36%, H 7.00%; for $C_{22}H_{28}O_6$, C 68.02%, H 7.27%; Found, C 68.17%, H 7.29%).

The infrared spectra of these compounds are shown in Figures 1, 2, 3 and 4.

We are indebted to Kyowa Fermentation Company and Takeda Pharmaceutical Company for kindly supplying the crude crystals of gibberellins.

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